AD-A221 528

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	7 MAY 90	Final

4. TITLE AND SUBTITLE

Production of Exocytic Vesicular Antigens by Primary Liver Cell Cultures.

5. FUNDING NUMBERS

G: NSF BCS-8919118 and Syracuse University Small Grants Program Grant No. 16

6. AUTHOR(S)

TIMOTHY F. MOSHIER
CPT, CM
US Army

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Department of Biology, Syracuse University
108 College Place
Syracuse, NY 13244-1270

8. PERFORMING ORGANIZATION REPORT NUMBER

N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Syracuse University, Department of Biology
Dr. James Smith (academic advisor)

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

N/A

11. SUPPLEMENTARY NOTES

Prepared in Cooperation with Dr. James Smith, Dr. Justine Garvey (both of Department of Biology, SU), Dr. Phillip Rice and Dr. Alan Freidman (both of School of Engineering SU)

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Available to the public. ~~DOD~~

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

This research demonstrated the feasibility of using primary mixed liver cells in a bioreactor for the production of a pure population of immunogenic vesicles. This work has shown that mechanically disaggregated cells from adult CD rats are capable of maintaining their viability, morphology and biochemical phenotype for prolonged periods of time (more than 19 days) without the addition of serum, mixtures of adhesins, corticosteroids, or dimethyl sulfoxide. A significant innovation for this work was the development of a microporous, gelatin-nylon cell carrier. When liver cells were encapsulated in sheets of the gelatin sponge, they aggregated into clusters with a morphology reminiscent of whole liver tissue. The gluataraldehyde-treated gelatin provided surfaces for cell attachment and is adaptible to a variety of bioreactor configurations

Especially significant is the finding that liver cells can reexpress endocytosed antigen in a vesicular form. Isolation of a pure population of antigen-containing vesicles

14. SUBJECT TERMS

Primary liver cell culture; antigen processing; vesicularized antigen; electron microscopy.

15. NUMBER OF PAGES

96

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

PRODUCTION OF EXOCYTIC VESICULAR ANTIGENS BY PRIMARY LIVER CELL CULTURES

by

TIMOTHY FRANCIS MOSHIER

B.A., State University of New York, College

at Oswego, Oswego, New York, 1981

ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirements

for the degree of Master of Science in Biology in the

Graduate School of Syracuse University

May 1990

Approved James E Smith

Date May 8, 1990

ABSTRACT

The high monetary and human costs associated with microbial diseases have created a demand for novel strategies to synthesize safe, effective and economical vaccines. The ideal vaccine would be an immunogenic agent capable of inducing a strong and specific response in recipients, would exhibit no significant side-effects, and could be produced inexpensively in pure form. This study proposes a new candidate for such a vaccine -- one or more of the species of vesicle-enclosed, processed antigens which are exocytosed from activated, antigen processing cells. However, before vesicles can be used on a large scale, a cell culture system must be devised to support large scale production of vesicularized antigen.

This research demonstrated the feasibility of using primary, mixed liver cells in a bioreactor for the production of a pure population of immunogenic vesicles. This work has shown that mechanically disaggregated liver cells from adult CD rats are capable of maintaining their viability, morphology and biochemical phenotype for prolonged periods of time (more than 19 days) without the addition of serum, or expensive mixtures of adhesins, corticosteroids, and dimethyl sulfoxide. Furthermore, it was demonstrated for the first time that a mixed liver cell culture is capable of executing the liver-associated function of clearing macromolecular and supramolecular antigens from its environment. A significant innovation for this work was the development of a microporous, gelatin-nylon cell carrier. When liver cells were encapsulated in sheets of the gelatin sponge, they aggregated into clusters with a morphology reminiscent of whole liver tissue. The glutaraldehyde-treated gelatin provided surfaces for cell attachment and is adaptable to a variety of bioreactor configurations.

Especially significant is the finding that liver cells can re-express endocytosed antigen in a vesicular form. Processed, vesicularized antigen is a potentially superior vaccine by virtue of it being: a) supramolecular and unable to pass the blood-brain barrier,

b) free of any whole pathogen, and c) a product of natural processes, making it a potentially strong immunogen. Isolation of a pure population of antigen-containing vesicles from the media is accomplished easily by centrifugation. The vesicles are uniform in size (300 ± 50 nm in diameter) and appear to have dense cores when stained with uranyl acetate and then visualized with transmission electron microscopy. Dynamics of antigen release have been investigated using enzyme-linked immunosorbent assays. These assays reveal that all the vesicularized antigen is shed on the fourth and fifth days after inoculation, suggesting that the vesicular antigen isolated here is related to the processed antigen released from the liver *in vivo*. A final and unexpected finding was that there were two classes of processed antigen -- the vesicular form and a smaller form which is released over the course of at least 12 days.

PRODUCTION OF EXOCYTIC VESICULAR ANTIGENS BY PRIMARY LIVER CELL CULTURES

by

TIMOTHY FRANCIS MOSHIER

B.A., State University of New York, College

at Oswego, Oswego, New York, 1981

THESIS

Submitted in partial fulfillment of the requirements

for the degree of Master of Science in Biology in the

Graduate School of Syracuse University

May 1990

Approved James E. Smith

Date May 8, 1990

ACKNOWLEDGMENT

I would like to thank the members of my committee; Dr. Thomas Fondy, Dr. Caroline Enns, Dr. Justine Garvey and Dr. James Smith for the valuable guidance they have given in the preparation of this thesis.

I would also like to thank the staff of the Ultrastructure Research Center of the State University of New York, College of Environmental Science and Forestry for their very generous assistance with electron microscopy and micrography; especially Dr. Robert Hanna, Mr. Jack McKeon and Mr. Arnold Day.

This research was supported by grant BCS-8919118 from the National Science Foundation and by grant No. 16 from Syracuse University Small Grants Program, April 15, 1989.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATONS

APC -- antigen presenting cell.

BSA -- bovine serum albumin (fraction 5).

cAMP -- cyclic adenosine monophosphate.

dH_2O -- distilled water (single).

DMSO -- dimethyl sulfoxide.

EGTA -- 1,2 - Di (2-aminoethoxy) ethane NNN'N' tetra-acetic acid.

ELISA -- enzyme-linked immunosorbent assay.

FDC -- follicular dendritic cell (of the popliteal lymph node).

HBSS -- Hanks balanced salt solution.

HBSS-EGTA -- HBSS, $-Mg^{2+}$, $-Ca^{2+}$, with EGTA.

hrs -- hours.

IgG -- immunoglobulin G.

IL-1 -- *interleukin - 1.*

LPS -- lipopolysaccharide-B (derived from *S. typhosa*).

MHC I -- major histocompatibility complex I.

MHC II -- major histocompatibility complex II.

min -- minutes.

moi -- multiplicity of infection.

mw -- molecular weight.

PBG -- PBS supplemented with albumin and gelatin.

PBS -- phosphate buffered saline.

pfu -- plaque forming units.

RNP -- ribonucleoprotein.

RSA -- rat serum albumin.

INTRODUCTION

The development of new strategies for the production of strongly immunogenic, safe vaccines is imperative if progress is to be made in the continuing campaign against microbial diseases. There remains a pressing need for novel vaccines against new agents as well as familiar pathogens such as *Streptococcus pneumoniae*, hepatitis B virus, *Plasmodium* spp. and dengue virus, which are creating tremendous burdens worldwide [32]. Most of the currently licensed vaccines utilize either attenuated live pathogens or inactivated pathogens [32], some of which cause immunopathological reactions in a significant fraction of the vaccinees [6]. The adverse side-effects may be a reaction to the altered pathogen itself, or to unrelated constituents in the vaccine menstruum [1]. The ideal vaccine must have the following properties: 1) ability to induce both humoral and cellular immunity, 2) ability to induce immunity equal in strength and duration to naturally acquired immunity, 3) no significant side-reactions, 4) no oncogenic or teratogenic properties, and 5) logistically simple to administer [31].

Testimony to the importance of obtaining ideal vaccines is the energy being spent in the search for new, and alternative types [9]. One approach is the use of recombinant DNA techniques to obtain antigenic determinants expressed by non-pathogenic microbial symbionts which occur naturally in the gut and on mucous membranes. Another method involves the use of synthetic peptides which mimic pathogens' immunogenic epitopes; this attempts to induce an immune response without using the entire pathogen. Also, some indirect immunizing strategies are being pursued such as the use of idiotype vaccines. These idiotypic vaccines are based on a complex sequence of events which are believed to take place in the immune system when an antibody's unique antigen-binding peptide sequence (the idiotype) stimulates production of another antibody directed against it (anti-idiotype). This idiotype-anti-idiotype reaction is thought to repeat itself, eventually stimulating antibody production against the antigen of interest.

The following experimental thesis is a fourth new alternative. It explores the feasibility of exploiting naturally occurring processes for new, simpler and potentially superior methods of vaccine production; namely, vesicularized-antigens produced by primary liver cell cultures.

This work describes for the first time a method to produce vesicular, processed antigen by a primary, mixed liver cell bioreactor. The term vesicular, processed antigen is used here to describe the product that arises when native protein antigen is taken up by animal cells, proteolytically modified and subsequently exocytosed in membrane-enclosed vesicles. In theory, a vesicular, processed antigen may be superior to other forms of vaccines: it would be capable of inducing a strong immunogenic response through naturally occurring mechanisms; the use of cell-free fragments from microbes would preclude the chance of attenuated pathogen undergoing reversion to wild-type virulence with subsequent disease in the recipient; packaging the degraded pathogen in submicron vesicles would prevent passage of the pathogen through the blood-brain barrier (thus preventing encephalitic reactions which can occur with some existing vaccines); since the vesicles are naturally exocytosed from the antigen presenting cells (APCs), they are easily seperated from the whole cells themselves and may be collected by simple centrifugation.

This research has demonstrated several important "firsts", including: 1) a cell carrier system which is easy to adapt to different bioreactor configurations; 2) demonstration that a mixed liver cell culture can maintain its viability and adult phenotype for prolonged periods of time without addition of serum, adhesins, corticosteroids, or dimethylsulfoxide (DMSO); 3) demonstration that antigen can be taken up and processed by liver cells *in vitro*; and 4) quantitation of the yield of processed antigen from the immobilized, cultured cells.

The design of the experiments relies heavily on literature regarding antigen processing, the role of the liver in the immune response, vesicle formation by cells, and manipulation and maintenance of primary liver cells in culture. The relevant literature is

reviewed below. The observations cited in the literature make the following predictions tenable: 1) the adult phenotype of liver cells (and thus their ability to conduct tissue specific processes) can be maintained in a culture system that uses mechanical disaggregation techniques to preserve the cells' natural extracellular matrix, 2) a mixed liver cell culture is capable of endocytosing supramolecular antigens, and 3) antigen processed by liver cells may be released in a vesicular form.

Antigen Processing.

Endocytic uptake, processing and presentation of antigen by specialized cells is perhaps the first step in the immune response [2, 9]. Macrophages, dendritc cells, and Langerhans' cells have long been recognized as primary APCs, and it was with macrophages that most of the work on intracellular mechanisms of antigen processing has been done. The macrophage model showed that foreign particles which had been endocytosed were subsequently degraded and denatured by endosomal enzymes [2]. Furthermore, it has been found *in vivo* and *in vitro* that macrophages which were exposed to antigens such as T2 bacteriophage and Glu-Ala-Tyr synthetic polypeptides of 50,000 mw covalently bound fragments of the degraded antigen to a molecule of RNA [24, 25]. This resulted in the formation of an immunogenic, 28S ribonucleoprotein (RNP). However, endocytosis of foreign particles is not required for RNP formation. Macrophages express 28S RNP on a constitutive basis, but specific antibody formation to a particular RNP occurrs only after exposure of macrophages to a particular antigen. Interestingly, the constitutive expression of a homogeneous population of RNPs, which become specifically immunogenic after exposure to a particular antigen, has also been demonstrated in the liver [22].

The Role of the Liver in Antigen Processing and the Immune Response.

In recent years the liver has been shown to play an important role in the immune system. The three major types of cells in the liver have been investigated extensively [20, 22, 28, 58], and they have been shown to possess many features in common with cells of

the immune system, including the internalization, processing and storage of antigen. The studies of J. S. Garvey and her co-workers, which extend over three decades, have detailed the liver's role in the immune response. They found that animals injected with soluble antigens (bovine serum albumin [BSA] and keyhole limpet hemocyanin [KLH]) processed and retained fragmented antigens in the liver for extended periods of time. However, after a second exposure of the cells to the antigen, the primary antigen was released from the liver and re-expressed in the spleen, and to a lesser extent, the lymph nodes [20]. Characterization of the antigen stored in the liver cells revealed that it had been processed by being partially degraded to a lower molecular weight form and then covalently linked to a length of RNA. This RNA-protein complex (RNP) is similar to one described for antigen-activated macrophages (in macrophages the RNA carrier was found to be structurally identical for all antigens [22]). Unlike macrophages though, the liver cells did not appear to use cell-to-cell contact for presenting the antigen to lymphocytes. Instead, they released the antigen into the sinusoidal spaces for transport to other organs and for further processing, similar to the lymph node model [63]. A striking feature of the processed antigen was its ability to induce much stronger immunogenic reactions than an equivalent amount of native antigen [28]. When liver RNP from an immunized rat was isolated and injected into other similarly immunized rats, the RNP effected a booster response equal to 5000 times the native antigen [22]. These findings suggested to the authors that the primary role for the liver in the immune response is antigen retention and heightened response after subsequent antigen challenges.

The liver is a highly vascularized organ composed principally of three cell types: hepatocytes (also known as parenchymal cells) which are responsible for synthesis of serum and bile proteins; Kupffer cells which are sinusoidal macrophages [9]; and endothelial cells, which line the sinusoids of the liver. Evidence indicates that the hepatocytes, Kupffer and endothelial cells all play important parts in the liver's immunological role. The clearing of antigen-antibody complexes has been mainly

attributed to the Kupffer and endothelial cells, perhaps with small amounts taken up by hepatocytes [59]. The hepatocytes are also responsible for the transfer of immunoglobulin A from the blood to the bile [59]. It has recently been shown that both Kupffer and endothelial cells are capable of acting as APCs [66]. They present on their surfaces class II major histocompatibility antigens (MHC II) necessary for interaction with T and B cells, and secrete interleukin-1 (IL-1), a T-cell growth hormone. At first, the storage and release of processed antigen in the liver was attributed entirely to the hepatocytes [20, 22], but later examination showed that the Kupffer and endothelial cells also act in this capacity [28]. In fact, the initial uptake of supramolecular antigens by the hepatocytes may require the presence of non-parenchymal cells. Kupffer cells are responsible for the endocytosis of macromolecular and supramolecular particles, while hepatocytes are restricted to endocytosis of smaller particles [70]. All these observations suggest that: 1) the liver plays a central role in the immune response, and 2) all three cell types may be required for antigen up-take and processing.

Extracellular Vesicle Formation.

Central to this research is the picture that the processed antigens will be exocytosed from the cells in membrane enclosed vesicles. This expectation arises from repeated observations that cultured cells release a variety of protein-containing vesicles into their environment (Table 1). One common example of vesicles exocytosed by cells are the large, heterogeneous "blebs" which are produced when cells have been exposed to either certain drugs or traumatic treatments such as low temperature [65]. Constitutive exocytosis of smaller, more homogeneous populations of vesicles by both normal and tumor cells in culture has been studied for two decades [64, 65, 68]. These "lipid-protein complexes", which are formed in a selective process, may be quite large, ranging from 300,000 to 50,000,000 mw as determined by gel chromatography [15, 65]. Particularly germane to this study is the finding that hepatocytes constitutively shed lipid-protein complexes

Table 1. Characteristics of extracellular vesicles.

Type of vesicle	Characteristics
Blebs	Large (>0.5 μm), heterogeneous, contain cytoplasm. Induced by drugs which interfere with cytoskeleton, and by trauma.
MHC associated vesicles	Small to intermediate (≈0.1μm), homogeneous population of vesicles constitutively shed by both normal and tumor cells.
Low density lipo-protein vesicles	Small (0.05 - 0.11 μm) homogeneous population of vesicles constitutively shed by hepatocytes.
Iccosomes	Intermediate (0.25 - 0.70 μm) homogeneous population of vesicles formed by FDC of the lymph nodes after secondary antigen challenge. Vesicles are progressively released over time to support the anamnestic response.

ranging in size from 0.05 - 0.11 μm in diameter. These hepatic vesicles appear to be the product of normal metabolic activities, and are hypothesized to be carriers for the delivery of lipoprotein to other tissues [11].

A direct role for extracellular vesicles in the activation of the immune system has been demonstrated with the follicular dendritic cells (FDC) found in the popliteal lymph nodes. These cells possess cytoplasmic processes which form interconnected beads termed "iccosomes" (immune complex coated bodies) [63]. Iccosomes range in size from 0.25 - 0.70 μm in diameter, contain processed antigen and present Ia complexes (MHC II) on their surface. The iccosomes act as reservoirs for the processed antigen while they are attached to the FDC, but once they are released, they become carriers for presentation of the antigen to other APCs. The FDCs are associated with the secondary antibody response, as opposed to the macrophage-mediated pathway which occurs after primary antigen challenge. In this alternative pathway, antigen-antibody complexes are transported on the surface of non-phagocytic, monocyte-like transport cells to the lymph nodes where the antigen is retained by the FDC. After the immune complex is internalized in the iccosomes, the iccosomes are released and dispersed. They may attach to Ia^+ (MHC II^+) germinal center B cells or become phagocytized by tingible body macrophages in the lymph nodes [63]. Parallels between this system of antigen up-take, storage and release, and the processing previously described in hepatocytes can be drawn easily [22]. It has been proposed that iccosomes may support the anamnestic response, i.e. immunological memory and heightened response upon exposure to a previously encountered antigen. We suggest there is an analogous system for presentation of vesicularized antigen to APCs in the liver.

Maintenance of Primary Liver Cell Cultures.

Three important criteria must be met in the design of a bioreactor for the production of vesicles by cultured liver cells. These criteria include retention of adult phenotype by vesicle producing cells for prolonged periods of time, a semi-rigid support that allows

manipulation of the culture system while protecting the cells from injurious shear effects, and an adequately porous cell carrier that allows for free passage of native antigen to the cells and release of vesicles into the media for collection.

Several factors are important to the maintenance of adult phenotype in cultured liver cells: cell-to-cell contact [26, 27, 45, 46], attachment surfaces which are equivalent to the basement membranes supporting cells *in vivo* [13, 41, 45, 47, 58], and hormonal and nutritional requirements [34, 35, 41, 45, 48, 50].

Physical contact between parenchymal and endothelial cells in culture has been shown to prolong viability and phenotype of hepatocytes in culture. The exact nature of the cell-to-cell contact requirement is not fully understood, although it involves neither intercellular exchange of materials [26] nor conditioning of the media by the cocultured cells. Rather, it seems to involve insoluble extracellular factors, perhaps extracellular matrix proteins [45]. This requirement for physical contact between cells, through as yet undefined structures, suggested that maintenance of a natural extracellular matrix might be a priority for long term culture studies. The most obvious way of preserving the natural extracellular matrix is by avoiding the use of proteolytic enzymes when harvesting cells.

Various adhesins and basement membrane proteins, such as collagen and fibronectin, have been found by many investigators to prolong differentiation and viability in primary cell cultures [41]. It has also been shown that liver parenchymal cells should be plated over the basement membrane proteins, and for optimal results, a second layer of protein should be precipitated over the cells to form a "sandwich" [13]. The sandwich configuration apparently mimics the *in vivo* environment by placing the transmembrane signal proteins against all facets of the cell [5]. Avoiding the collagenase perfusion normally used to isolate liver cells obviously would help maintain the extra-cellular structures which normally envelope the cells and would preclude the need for supplementing the culture with matrix proteins.

Collagen (or a collagen derivative) is a prime candidate for use as a cell support material in an immobilized cell bioreactor because of its proven ability to provide an attachment surface for mammalian cells. As typically used in culture work, collagen and its derivatives are gelatinous precipitates, and some modifications of the gel must be made to provide the rigidity and malleability required for a 3-dimensional cell carrier system in a bioreactor. Precipitating collagen onto nylon mesh supports provided an infrastructure for the collagen gel, which allowed investigators to study the maintenance of liver cells on floating collagen membranes [47]. Recently, a bioreactor has been described using bacterial cells immobilized in gelatin and coated onto a nylon mesh, with subsequent cross-linking of the gelatin and cells by glutaraldehyde [3]. The combination of chemical cross-linker and semi-rigid support provides a flexible and porous biocatalyst surface. However, the extreme conditions described for the construction of this carrier would kill mammalian cells, unless alternative, milder cell carrier construction protocols could be found.

The complete nutritional requirements for a long-term mixed liver cell culture are not yet fully known, but investigators are aware of miscellaneous essential nutritional and hormonal supplements, and some additives which appear to prolong viability and differentiation. Pitot and others reported that insulin is absolutely required for the retention of viable cells in adult hepatocyte cultures [50]. The hormone appears to stimulate cell aggregation and formation of polyribosomes [47], and to induce differentiation [18]. It has also been found that primary liver cell cultures maintained an adult phenotype longer when kept on floating collagen gels than when anchored to the bottom of collagen coated plates [47]. The longevity was presumed to be due to better oxygenation of the cell clusters. A number of investigators have supplemented their liver cell culture media with hydrocortisone [13, 26, 46]. Corticosteroids, however, may act as immunological suppressants [9], and so may not be useful in achieving the goals of this research. Dimethyl sulfoxide (DMSO) induces differentiation in several cell systems, including hepatocytes [34,35], although it results in cytoskeletal aberrations and misshapen

mitochondria [16, 45]. Release of membrane-coated antigen presumably would require an intact and functioning cytoskeleton. DMSO's demonstrated ability to interfere with cytoskeletal structures makes its use undesirable in our studies.

Supplementation of the bioreactor medium with serum is contraindicated in numerous papers. It has been known for a considerable period of time that prolonged exposure of hepatocytes to serum results in their premature death [19]. Differential loss of one or more classes of liver cells from the bioreactor could possibly destroy the interactive antigen-processing mechanisms. Also, the presence of serum in the vesicularized antigen preparations could possibly lead to "serum sickness" side-effects in vaccinees [1].

An unusual supplement used in this work is the bacterial endotoxin, lipopolysaccharide-B (LPS). LPS can have very different effects on different types of cells. For example, phagocytosis by macrophages may be significantly stimulated, presumably due to activation of membrane receptors. Hepatocytes, on the other hand, experience increased activity of cyclic adenosine monophosphate (cAMP) mediated functions, which results in glycogen degradation and release [38]. In the immune system, LPS can induce production of interleukin-1 (IL-1), a T-cell growth hormone [66]. Because of its wide range of effects and adjuvant-related activity, it was decided to evaluate how LPS might influence the ability of liver cells to internalize, process, and release antigen *in vitro*.

MATERIALS AND METHODS

Materials.

Animals: All animals used in this work were four to five month old adult CD rats: Harlan Sprague Dawley, INC., Indianapolis, IN.

253 μm nylon mesh: TETKO, Elmford NY.

1 M $MgCl_2$ in 10 mM HCl: Sigma Chemical Company, St Louis, MO.

96 well micro-titer plates (EIA Plates), and adhesive covers for 96 well plates: Flow Laboratories, McLean, VA.

Alkaline Phosphatase Substrate (p-nitrophenol phosphate, disodium) 5 mg tablets: Sigma Chemical Company, St Louis, MO.

Anti-Rabbit IgG Alkaline Phosphatase Conjugate: Sigma Chemical Company, St Louis, MO.

Anti-Goat IgG Alkaline Phosphatase Conjugate: Sigma Chemical Company, St Louis, MO.

Artek vertical beam plate-reader (for reading ELISA plates): Artek Division of Dynatek Corp., Chantilly, VA.

AuroProbe EM Goat-anti-Mouse, Janssen Life Sciences Products: Amersham Corp., Arlington Heights, IL.

Bacteriophage T2, strain 11303-B2: American Type Culture Collection, Rockville, MD.

Bacto-Lipopolysaccharide-B, (*S. typhosa* 0901) (LPS): DIFCO Laboratories, Detroit, MI.

Carbon/Formvar Coated Grids, 300 mesh: Polysciences, Inc., Warrington, PA.

Centriflo Centrifugal Concentrators: Amicon Division, W. R. Grace & Co., Danvers, MA.

DIFCO Special Agar - Noble: DIFCO Labs, Detroit, MI.

Eschericia coli B, strain B11303: American Type Culture Collection, Rockville, MD.

Gel Filtration Chromatography Medium: Spectra/Gel A6, Spectrum Medical Industries, Inc., Los Angeles, CA.

Gelatin EIA Grade: BioRad Laboratories, Inc., Richmond, CA.

Gelatin used for cell carriers, Sigma Cell culture tested, 300 bloom, porcine: Sigma Chemical Company, St Louis, MO.

Glutaraldehyde used for cell carrier construction: 25% by weight, Kodak, Rochester, NY.

Glutaraldehyde, E. M. Grade: Polysciences, Inc., Warrington, PA.

Goat-anti-Rat Serum Albumin, Cappel Immunochemicals: Organon Teknika Corp., West Chester, PA.

IntenSE M Silver Enhancement Staining Kit, Janssen Life Sciences Products: Amersham Corp., Arlington Heights IL.

Keyhole Limpet Hemocyanin (KLH): A gift from J. S. Garvey, Syracuse University, Syracuse, NY, prepared in accordance with procedures given in 21.

L-15 tissue culture medium: GIBCO, Grand Island, NY.

Linbro 7X detergent: Flow Laboratories, McLean, VA.

Mouse Monoclonal-anti-Rat Ia (Polymorphic) and Mouse Monoclonal-anti-Rat MHC I: Bioproducts for Science, Inc., Indianapolis, IN.

Nickel E. M. Grids, 75/300 mesh: Polysciences, Inc., Warrington, PA.

Normal Goat Serum, Janssen Life Sciences Products: Amersham Corp., Arlington Heights, IL.

Polybed 812 (aka Epon 812): Polysciences, Inc., Warrington, PA.

Polycarbonate Membranes, 0.1 μm pore size: Poretics Corp., Livermore, CA.

Polycarbonate Oak-Ridge style centrifuge tubes, 30 ml: Nalge Company, Rochester, NY.

Quantigold: Diversified Biotech, Newton Centre, MA.

Rabbit-anti-KLH: A gift from J. S. Garvey, Syracuse University, Syracuse, NY.

Rat Albumin, FR V: ICN ImmunoBiologicals, Lisle, IL.

Semi-micro Disposable Polystyrene Cuvettes: Fisher Scientific, Rochester, NY.

Spectra/Por Dialysis Tubing #2: Fisher Scientific, Rochester, NY.

Tryptone and Yeast Extract: DIFCO Labs, Detroit, MI.

Tween 20: BioRad Laboratories, Inc., Richmond, CA.

Methods.

Isolation of Liver Cells.

The goal of the surgical and tissue disaggregation processes was to provide a population of rat liver cells which were in small clusters, sheets, or in individual cells. Several methods of tissue disaggregation were tried, including thin slicing, maceration using a loose fitting tissue homogenizer, and mechanical disaggregation using nylon screens. Use of proteolytic enzymes was considered undesirable since one of the goals was to preserve the integrity of the proteins in the extracellular matrix as much as possible. The most successful method used minced sections of the tissue which were passaged through increasingly finer nylon meshes until the desired degree of dissociation was achieved. The surgical procedures were designed to excise the liver aseptically with a minimal amount of handling; blood and lymph cells were removed by differential centrifugation and chemical lysis. Details of the surgical and tissue disaggregation protocols are contained in appendix B.

Prior to the actual harvesting of cells, all glassware used in tissue handling and culture were cleaned thoroughly to remove endotoxin. Bacterial endotoxins may cause a range of cellular responses, some of which are deleterious to liver cells. Also, the effects of the endotoxin LPS were being studied, and contaminating endotoxin would interfere with these measurements. Cleaning of glassware was accomplished as follows: the glassware was soaked in a 5% sodium hypochlorite solution; washed using Linbro 7X neutral detergent; and rinsed 4 times with tap water, twice with metal distilled water, and twice with glass distilled water. The glassware was then dried, inverted, in a 180° C oven for 4 hrs before sterilization at 121° C for 20 min [18].

Surgery: Rats were anesthetized with ether, shaved ventrally, and thoroughly washed with 70% ethanol to provide a sterile working area. Exaggerated incisions were made along the midline and transversly to facilitate manipulation of internal organs, and provide a large, sterile working area. After the vena cava had been clamped above and

below the liver to prevent the flow of blood, the portal vein was cut, and the lobes of the liver were excised and immediately transferred to a sterile petri plate containing HBSS (Hanks balanced saline solution), -Mg^{2+}, -Ca^{2+}, with EGTA (1,2 - Di [2-aminoethoxy] ethane NNN'N' tetra-acetic acid) (see Appendix A), washed and placed on ice. Excised tissue was kept on ice for the shortest possible period of time to prevent cold induced damage to the cells.

Tissue disaggregation: The tissue disaggregation procedure described here was based on a technique described elsewhere [18], but modified to use red blood cell lysis procedures [10], and low speed centrifugation [42]. Basically, the lobes of the liver were minced into 3 mm^3 pieces using loose-jawed scissors, and washed with buffer. By means of a plunger from a 50 ml syringe, the pieces of liver tissue were forced through a 1 mm nylon mesh (window screen variety), which had been stretched across the top of a 250 ml beaker. The tissue was wetted with HBSS, -Mg^{2+}, -Ca^{2+}, with EGTA (HBSS-EGTA) at all times to prevent desiccation. This first suspension of coarsely disaggregated tissue was then forced through a 273 µm nylon mesh, transferred to sterile, disposable 50 ml centrifuge tubes, and centrifuged for 4 min at 100Xg, 4° C to pellet whole cells. The pellets were then suspended in 10 - 15 ml of NH_4Cl buffer (see Appendix A) and incubated on ice for 8 min to lyse red blood cells. The tubes were then slowly filled with HBSS-EGTA [10], and centrifuged at 100Xg, 4° C for 4 min. The pellet of relatively pure liver cells was then washed a final time in L-15A medium (see Appendix A).

Rationale for use of medium and buffer: In the above discussion, the use of HBSS, -Mg^{2+}, -Ca^{2+}, with EGTA has been mentioned; the rationale for using it is simply that calcium - and magnesium - free HBSS plus EGTA washes sequester divalent ions required by lytic enzymes, as well as maintain physiological pH and osmolarity for the cells.

L-15A medium is an adaptation of a formula which had proved useful in longevity studies of hepatocyte cultures [26]. The basic Leibovitz L-15 medium is well suited for

large bioreactors. It is formulated for use at ambient CO_2 concentrations [18] and supplies galactose as an energy source. The galactose can be metabolized in the liver into UDP-galactose -- a substrate for glycoprotein synthesis as well as an intermediary for entry into the glucose metabolism pathway with low acid production [44]. The basic L-15 medium contained several supplements: insulin to enhance aggregation of the cells [47] and to maintain biochemical differentiation [18]; HEPES and sodium bicarbonate to assist in maintaining extracellular pH and CO_2 concentrations [18]; glucose as an alternative source of energy and pyruvate (pyruvate helps to maintain intracellular CO_2 concentrations); and transferrin, to provide iron in a form usable by the cells [40]. Transferrin has also been found to act as a growth stimulant in hepatoma cell cultures [57].

Alternative disaggregation methods: Other cell harvesting methods were tried, but found unsuitable for various reasons. Thin slicing of liver tissue using a variety of knives and tissue immobilization techniques [67] failed because the tough connective tissues and basement membranes of liver made it impossible to achieve clean, uniform sections 10 - 15 cells thick (thin sections allow free passage of nutrients to all the cells in the section). The second method involved macerating 3 mm^3 fragments of tissue suspended in PBS into small clusters, using a loose fitting manual Vitro-A tissue homogenizer. Problems associated with this technique included both a low viability count and the small amount of liver that could be handled at one time (about 4 g).

Construction of the Cell Carrier.

A very successful system for immobilizing dense populations of liver cells was developed using a bacterial bioreactor as a model [3]. The bacterial bioreactor used glutaraldehyde to cross-link gelatin-bacteria suspensions to create porous, semi-rigid supports containing embedded, inactivated cells. Although the construction protocol had to be modified to support live cell culture, the properties of the bacterial cell support were ideal for our purposes. The original protocol was modified by glutaraldehyde cross-linking of the gelatin on nylon mesh, and washing excess glutaraldehyde from the system before

seeding the cells. It was found that low temperature gelation of the gelatin/glutaraldehyde mix produced a resilient sponge with 10 - 20 μm cavities (figure 3C) which remained flexible when hydrated.

The protocol for fabricating the carriers was quite simple and required no specialized equipment. Nylon mesh, 253 μm, was cut in 12.5 cm x 6.5 cm strips, washed in 95% ethanol, then metal distilled water, and finally glass distilled water. The strips of mesh, 8" x 11" pyrex baking dishes covered with aluminum foil, and various mixtures of gelatin were sterilized at 121° C for 20 min. After the baking dishes had cooled to room temperature, they were lined with ethanol-washed Saran-Wrap (the plastic film lining was required to provide a non-stick surface for the gelatin as it cured), dried in a 100° C oven for several hours, then transferred to a freezer to chill. The strips of sterile nylon cloth were laid in the chilled baking dishes; 3 ml of glutaraldehyde solution was mixed with 3 ml of gelatin solution in a sterile test tube, immediately poured over one of the strips and spread evenly across it with a flamed glass spreader. After the nylon strips had been coated, the baking dishes were returned to the -15° C freezer and left to gel over-night. At least 4 hrs prior to seeding cells on the carriers, the gelatin-coated nylon mesh strips were removed from the freezer, two of them stapled together along three sides to form an envelope, and washed with sterile PBS (see appendix A) to remove any residual glutaric acid.

Other carriers which had been evaluated: Several cell carriers were evaluated before developing the gelatin coated nylon mesh, and although none proved useful, the failures are instructive. Attempts were made to covalently attach the cells directly to nylon mesh activated with glutaraldehyde. The protocol had originally been developed for the attachment of enzymes to nylon tubing [60], but worked badly for cells. The cells rapidly and preferentially attached to each other to form large aggregates, leaving only several cells in the aggregate available to attach to the nylon. This left the weakly attached cell masses susceptible to shear forces. Other methods of attachment which were tried included

collagen coated onto glutaraldehyde-activated nylon mesh, collagen precipitated onto nylon mesh using NaOH [58], precipitation of collagen and gelatin through 10X medium onto nylon mesh [13], and simple air drying of collagen onto nylon [47]. These methods produced either loose, flocculent precipitates, or brittle films. It was clear that encapsulation was needed to protect the cells and allow time for them to adhere to the carrier. It was also obvious that mechanically disaggregated cells would adhere to glutaraldehyde-treated gelatin as well as to native collagen.

Albumin Assay.

Albumin is a liver-specific protein, and its secretion by hepatocytes in culture may be used as a measure of the cultures' viability and differentiation [13, 26, 34]. Two methods of determining albumin content in the spent media were tried: preferential binding of phenol red to albumin [43], and the more sensitive enzyme-linked immunosorbent assay (ELISA). The first method, which entailed fractionating phenol red-supplemented spent media on a Sepharose column, proved too insensitive for this work. Fortunately, the commercial availability of anti-sera against rat serum albumin (RSA), combined with the sensitivity and specificity of the ELISA, made it a convenient test for culture fitness.

The albumin/ELISA involved a three-step reaction scheme to quantitate the albumin released by the cells into the spent media, these steps involved: (i) coating the wells of the microtiter plate with proteins from the spent medium, (ii) adding anti-RSA to the protein-coated wells and allowing selective binding with any RSA in the first protein coat, and (iii) adding an alkaline phosphatase-conjugated anti-IgG specific for the first antibody to form the third protein coat. The concentration of enzyme in the third coat was directly proportional to the concentration of RSA in the spent media. Direct quantitation was possible by measuring the absorbance of the hydrolyzed substrate (p-nitrophenol phosphate) at 405 nm. A detailed protocol is listed in Appendix B.

Determination of Antigen Uptake by Liver Cell Cultures.

The optimum choice for an antigen in this assay is one which is both strongly immunogenic (high molecular weight and phylogenetically distant form the host [9, 22]) and easily assayed. Coliphage T2 was chosen as the antigen for this study because it has a high molecular weight ($\approx 2 \times 10^8$ mw), is foreign to the rat, and can be quantitated easily by plating the free phage on its host *E. coli* B.

Antigen uptake by the liver cells was assayed by counting the phage remaining free in the spent media after 24 hrs. Several control roller bottles were incorporated into the antigen uptake experiment to determine the percentages of free phage lost to endocytosis by the liver cells and to nonspecific affects. All of the control roller bottles contained L-15A medium and phage. Selected control bottles were supplemented with one or more of the following; 10 μg/ml LPS, the cell carrier, and 10% calf serum supplemented medium.

Preparation of phage stock: About 5000 to 7000 phage per liver cell was determined to be a reasonable ratio for inoculation of the tissue cultures. To achieve the concentration of phage required for this ratio, high titer phage stocks were prepared by inoculating an *E. coli* B culture at moi = 5, which caused delayed lysis and increased yields of phage per bacterium [17]. Liver cell cultures were inoculated with T2 diluted in tryptone broth (Appendix A).

Gel Filtration and UV Spectrum Analysis of Vesicles.

In similar work regarding shedding of membrane-associated alloantigens, it was found that pure populations of supramolecular complexes could be separated from spent media by using agarose bead columns with high molecular weight cut-offs [15]. Spent media from liver cell cultures was similarly fractionated to provide a pure population of vesicles for UV spectral analysis. To concentrate the vesicles before loading the column, aliquots of spent media from day 4 cultures were concentrated using Amicon Centriflo centrifugal concentrators. Five ml aliquots were loaded into the filter cones, and then centrifuged for 40 min at 750Xg, 4° C. One ml of retentate, which contained the vesicles,

was left in the cones after the centrifugation (5 X concentration). The fractionation column (1 cm x 30 cm, Spectra/Gel 6 ($4x10^6$ mw exclusion limit) was an agarose gel with a flow rate of 8 - 10 ml/hr [49]. One ml fractions were collected. Fractions were initially screened for absorbance at 280 nm and 260 nm, and those showing the most activity were analyzed for absorbance over the spectrum 360 - 230 nm, with readings made at 5 nm intervals.

Photomicrography.

Samples of the carrier system and cells in culture were examined by light-microscopy to determine structural details of the cell carrier, gross histology of the mixed liver cells in culture, and whether or not cells were adhering to the carrier. Unfixed samples of cell carriers from 3 week old cell-free control bottles, and samples of cell loaded carriers, also from 3 week old cultures, were examined in transverse section with an inverted phase-contrast microscope. The 1.0 - 1.5 mm thickness of the whole carrier sheets required splitting the double-sheet envelopes in half so that only one gelatin coated nylon mesh was observed at a time. This had little apparent affect on the histology of the cultures, since the cells adhered predominantly to only one of the sheets. Cross-sections of the sandwich required embedding in Epon 812 resin because of the nylon fibers. Samples used for cross-sectioning were (a) fixed in 2.5% glutaraldehyde/ 2% formaldehyde in 0.1M sodium cacodylate buffer, pH 7.2, with 1 mM $CaCl_2$; (b) post-fixed with 1% osmium tetroxide; and (c) dehydrated through a series of ethanol solutions. After the ethanol had been displaced with propylene oxide, the samples were infiltrated with Epon 812 and polymerized at 65° C for ≈ 70 hrs [12]. A detailed fixation, dehydration and embedment protocol is listed in Appendix B. Sections (6 μm thick) were cut on a Sorvall Porter-Blum 2B ultramicrotome using glass knives. For light microscopy the Epon sections were stained with toluidine blue prior to observation (4.8% in 15% borax [$Na_2B_4O_7*H_2O$]) [12].

Transmission Electron Microscopy.

A major objective of this study was to determine if processed antigens were exocytosed in a vesicular form and what were the vesicles' physical characteristics. Electron microscopic techniques offered the most direct means using both thin sections and staining of whole vesicles from antigen-activated cells. Gold-conjugated immunolabelling was used to locate specific antigens in the sections. All samples were viewed with an RCA EMU-4 transmission electron microscope, 100 kV accelerating voltage.

Ultrathin sections: The relatively small (200 - 300 μm estimated size), delicate vesicles presented a handling problem due to the numerous steps required for fixation, dehydration and embedding. To overcome this problem, the pelleted vesicles were embedded in 1% agar [12]. The embedding protocol has been detailed elsewhere [23], and will be briefly summarized along with the centrifugation procedures used to collect the vesicles. The vesicles were resuspended using a Vortex mixer and centrifuged at 100Xg for 5 min, 4° C to pellet large cellular debris. The supernatant was transferred to sterile, 30 ml Oak-Ridge tubes, and centrifuged at 22,000Xg, 4° C, 40 min using an angle head rotor. The pellet was washed in 1 ml of sterile filtered PBS and resuspended in 1 ml of the same buffer. The suspension was then warmed to 45° C and mixed with 0.15 ml of 7% noble agar at 45° C. The mixture was immediately poured onto a chilled microscope slide, allowed to set, and cut into 1 mm^3 cubes.

Fixation, dehydration and Epon embedding of the agar cubes was the same as that described for tissue culture specimens, but with one exception -- the glutaraldehyde/formaldehyde fixation time was 48 hrs at 4° C. Thin sections from the vesicle blocks were mounted on nickel grids, since the periodic acid treatment used in the immunolabelling procedure (see below) prevented the use of copper grids.

Ultra-thin cross sections that were not going to be used for immunolabelling were stained with uranyl acetate (saturated in 50% ethanol, pH 4.0 to 5.0, adjusted with NaOH), and lead citrate (0.5% aqueous solution). The staining solutions were stored at 4° C and

filtered through 0.2 μm syringe filters prior to use. Sample sections were floated on drops of staining solution for 15 min at room temperature, with thorough washing between and after stains [12].

Immunolabelling of ultrathin sections: Immunolabelling of vesicles was used to determine: (i) the presence of the KLH antigen used to inoculate the cultures, and (ii) presence of major histocompatibility complex antigens class I and class II (MHC I and MHC II) on vesicle membranes. Simultaneous localization of KLH and MHC was done through the use of antibodies labelled with different sizes of gold particles. The anti-KLH antisera was from rabbits; the anti-MHC monoclonal antibodies were from mouse hybridomas. KLH was detected with anti-rabbit IgG conjugated to 5 nm gold particles; MHC I and II could be simultaneously detected with anti-mouse IgG conjugated to 10 nm gold particles. The protocol used was a modification of the method suggested by the supplier of the gold-conjugated antibody reagents (Appendix B). The principle exception to the manufacturer's instructions was the etching of sections for 1 hour in saturated sodium metaperiodic acid. Etching was required to displace osmium tetroxide from the epitopes, and allow antibody-antigen reactions to occur [54]. Negative controls for the immunolabelling of vesicle thin sections consisted of normal rabbit serum diluted 1:1000 in place of the anti-KLH antiserum, and PBG (PBS supplemented with albumin and gelatin, (see Appendix A) in place of the anti-MHC monoclonal antibodies.

Negative staining of whole vesicles: Rapid negative staining of vesicles was a modification of previously described protocols [30, 37]. Vesicles were concentrated using the differential centrifugation techniques described above, then resuspended in PBS. 1 ml of vesicle suspension was mixed with 1 ml of 1% uranyl acetate (dissolved in PBS, pH 7.4), and a drop of this was immediately placed on a carbon/formvar coated grid with a pasteur pipette. After 2 min, the excess liquid was slowly removed from the grid using the corner of a wedge shaped piece of filter paper, and then immediately viewed on the

transmission electron microscope. As a control, a drop of 1:1 PBS: stain was visualized on the microscope so that crystals from the stain would not be confused with vesicles.

En bloc immunolabelling of vesicles was attempted for a clear indication of whether the vesicles possessed MHC antigens on their external surfaces. However, the numerous washes required to avoid false positives resulted in complete loss of the vesicles.

Quantitation of Exocytosed Antigen.

The final goal was to quantitate the vesicular, antigenic product that was central to this work. Two methods were employed -- counting the vesicles directly, and analyzing the concentration of processed antigen exocytosed into the media.

Immuno-assay of vesicular antigen: ELISA methodology was used to quantitate antigen exocytosed by the cells, determine the dynamics of antigen release , and determine if sensitization of cells by LPS would have any significant effect on the release of processed antigen. Vesicles were concentrated according to the method used in electron microscopy with two modifications. First, the spent media was centrifuged at 70,000Xg to compact the vesicles tightly and to bring down low density vesicles (KLH requires settling at 78,000Xg [21]. Second, instead of suspending the vesicles in PBS, about 1 ml of L15A media was left in the centrifuge tube and used to resuspend the vesicles. To eliminate any possibility of the vesicle membrane interfering with the ELISA, vesicles were lysed with 0.5% Triton X-100 (a non-ionic detergent) for 30 min at room temperature. The vesicle suspensions were then dialyzed for 24 hrs against PBS supplemented with 0.01% sodium azide at 4° C, with one change of PBS [15]. The ELISA was generally set up in accordance with the protocol described for the assay of albumin in spent media, but with the following changes; the primary antibody (rabbit-anti-KLH antisera) was only diluted 1:200, and incubation time with the substrate buffer was extended to 2 hrs because of the low reactivity in the alkaline phosphatase conjugate. Controls in this set of experiments included cultures inoculated with T2 bacteriophage to determine if there was any reactivity

with conditioned medium from non-specifically activated cultures, and a bottle containing only a cell carrier and KLH to determine the carry-over of KLH after media exchanges.

Direct quantitation of vesicles: Although vesicles are submicroscopic, it was thought that fluorescent staining of them might enable direct quantitation [53]. Spent media was filtered through a 0.1 μm polycarbonate filter to concentrate the vesicles and distribute them across one plane; the vesicles were then stained with acridine orange (pH 3.8), rinsed, dehydrated, and observed using oil immersion objectives and an epifluorescent microscope. The vesicles were at the limits of resolution, and the background fluorescence caused by the polycarbonate filter made it impossible to obtain accurate counts. Because of this, all quantitation was made only in terms of the concentration of antigen exocytosed into the medium as described above.

Preparation of KLH from crude extracts: KLH used in these experiments was clarified from a crude extract according to a previously described protocol [21] to yield a relatively pure solution of antigen. Crude extract was (a) resuspended in 15 ml of PBS with gentle stirring overnight at 4° C; (b) centrifuged for 30 min, 1400Xg, 4° C to precipitate any gelatinous contaminants; (c) filtered through a #1 Whatman filter; (d) dialyzed against PBS for 24 hrs (3 changes of dialyzate); and (e) concentrated by centrifugation, 2 hrs, 78,000Xg, 4° C. The concentration of KLH was compared using both the biuret [21] and Quantigold assays (protocol according to manufacturer's instructions, see Appendix B).

RESULTS

Immobilization and Maintenance.

Immobilization: After several trials, a gelatin-nylon based cell carrier was developed which provides an encapsulating, porous, semi-rigid support for long-term maintenance of cell cultures. The first effort was to coat nylon screen with a fibrous, gelatin mat that was fine enough to retain the cells. Gelatin and glutaraldehyde were combined in different concentrations and observed microscopically to judge the coarseness of the mesh which formed. The combinations initially judged best were: 2% gelatin mixed with an equal volume of 6% glutaraldehyde, and 6% gelatin mixed with an equal volume of 6% glutaraldehyde. These combinations were further evaluated for adaptability in roller bottle culture as two layer (two gelatin coated nylon sheets stapled together) and single layer carriers seeded with cells (Table 2).

From the performance results summarized in table 2, it can be seen that double sheets of 2% gelatin: 6% glutaraldehyde (carrier II) made the best carriers. A double layer of screen coated as described afforded the cells a stable substrate and maintained itself against the sides of horizontal roller bottles turning at a rate of 1 revolution per minute. Cells within the fabric envelope were protected from damage by liquid phase shearing. The other carriers collapsed, rolled up, and prevented the distribution of media across the entrapped cells. The gelatin mats of single sheet carriers tended to fragment after a couple of days in culture, presumably due to the ends of the carriers curling over and scraping along the middle of the carrier strips.

Infrastructure of the mixed cell culture system: Initially, the structure of the gelatin matrix in the cell carriers was conceptualized as a net-like arrangement of coarse fibrils (hence the term "mesh" that has been used to this point). Also, the first observations of the glutaraldehyde-treated gelatin carriers by bright-field microscopy suggested that a net of fibrils did exist. Further examination revealed a much more complex structure, however.

Table 2. Cell carrier[a] performance.

Carrier	Construction Features	Performance in Culture
I.	2% Gelatin: 6% Glutaraldehyde Single Sheet.	Portions of carrier sheared away from nylon support. Lacked rigidity. Loss of cells.
II.	2% Gelatin: 6% Glutaraldehyde Double Sheet.	Cells were retained in mesh. No shear problem. Maintained carrier configuration against bottle.
III.	6% Gelatin: 6% Glutaraldehyde Single Sheet.	Portions of carrier sheared away from nylon support. Lacked rigidity. Loss of cells.
IV.	6% Gelatin: 6% Glutaraldehyde Double Sheet.	Lacked rigidity.

[a]All carriers were constructed with 253 μm nylon mesh support in accordance with protocol given in methods section.

When the gelatinized cloth was observed at high magnification by phase contrast microscopy and when seen in cross section, its true sponge-like structure became clear.

Figure 1A shows a transverse section of a carrier which had been used as a cell-free control for 3 weeks. The double-sheet envelope was split into individual sheets prior to observation because the thickness of the carrier sandwich would create too many diffraction patterns and confuse the picture. The surprising revelation in this figure was that the gelatin not only formed cords but also fenestrated sheets between the cords.

Further clues to the structure of the carrier can be found by studying cell-loaded carriers. Figure 1B shows the tip of an extended cell cluster, in which one of the cells appear to refract the light differently through different areas of the same cell (arrow head). Close inspection reveals that the cell is straddling the edge of a sheet of gelatin. It also appears as if the gelatin overlaps the nylon fibers in at least some of the places. All of this suggests that the carrier has considerable depth and surface area.

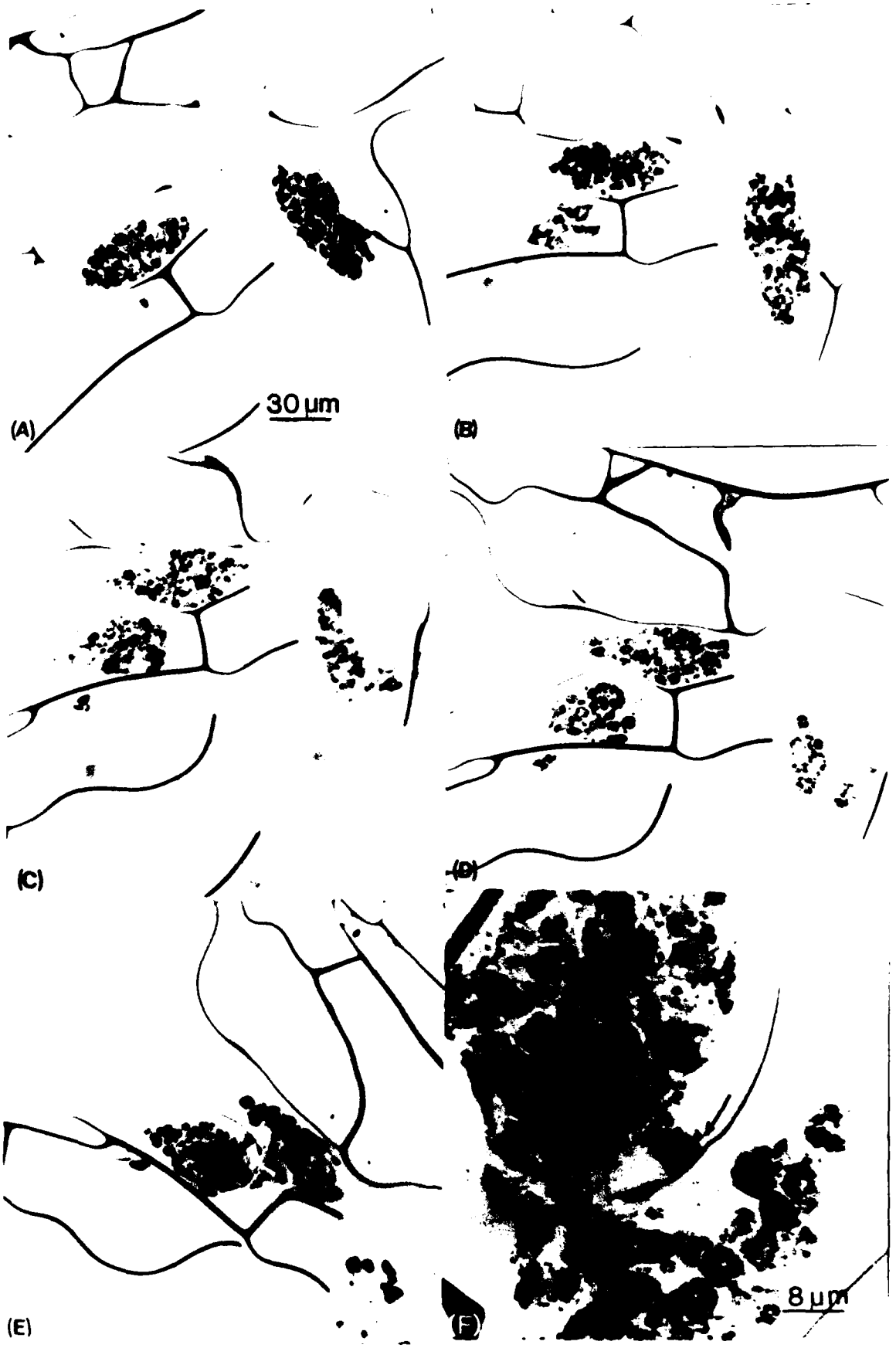
Figure 1B also gives some indication of the vitality of the cells in culture. Most of the cells have retained a cuboidal shape (which is typical of hepatocytes *in vivo*) as well as dense nuclei and a granular appearance to the cytoplasm. All of these characteristics are indicative of healthy, differentiated cells [45].

The planar views of the culture suggested a deep carrier with a three-dimensional, parenchymal-like structure comprised of cell aggregates. A more accurate picture of the carrier comes from cross-sections of the culture. Figures 2A through 2E are serial cross-sections through a 4-day old culture. It's obvious that the carrier is not a two dimensional sheet of gelatin, but rather a microporous sponge. As the point of focus moves through the sections, some chambers formed by the gelatin become narrow and disappear, while some gelatin sheets can be seen growing towards each other and fusing to form new chambers. Although not shown in these figures, the chambers of the sponge eventually open up to both the interior of the sandwich as well as to the exterior sides of the carrier. These figures also confirm the parenchyma-like structure of the cell aggregates,

Figure 1. Gelatin-coated nylon screen for immobilizing liver tissue culture cells. 1A, Transverse view of single sheet of the gelatin-nylon cell carrier taken from the cell free control bottle after 3 weeks of incubation is shown in transverse view. Chilled, glutaraldehyde-treated gelatin formed sheets (S) and cords (C). Two nylon fibers (N) are seen crossing at the center of the micrograph. Magnification = 900X. 1B, Transverse view of a 3 week culture seeded at a density of $\approx 10^6$ cells/cm^2. The section photographed represents the end of a thick cluster of cells. At the bottom of the figure is a dense cell cluster (Cc). N; nylon fiber, magnification = 1,010X. Both photomicrographs were made using medium dark phase contrast microscopy.


S
N
(A)
10 µm
N
(B)

Figure 2. Serial cross-sections (A - E) of a 4-day culture. The dark inclusions in the cells are lipid droplets stained with osmium tetroxide. Note how the right-hand cluster of cells tapers off, while the left cluster becomes increasingly larger, eventually joining with the center cluster. Magnification = 330X in figures 2A - 2E. Figure 2F is a higher magnification view of 2E showing cell arrangement. Note the cell apparently anchored to the carrier (arrow) (2F). Magnification = 1320X.


30 µm
(A)
(B)
(C)
(D)
(E)
(F)
8 µm

which is reminiscent of the liver with its many sinusoids and bile cannulae. The cell aggregates tend to be flattened ellipsoids, so that there are only 1 - 2 cells between the most interior cells of the aggregate and the medium. The aggregates provide extensive intercellular contact while maintaining adequate access to the aerated medium by each cell. Figure 2F is a high magnification view of one of the cell aggregates. In this figure the parenchymal structure is easily seen, but more importantly, cell attachment to the carrier is clearly indicated. The dark bodies in the polygonal cells (presumably hepatocytes) are lipid droplets densely stained by osmium tetroxide.

An overall view of the culture system is depicted in figure 3. A cartoon (3A) shows how the freshly disaggregated cells are distributed into the microporous sponge formed by the gelatin. Phase contrast micrographs show in transverse view how the cells tend to aggregate into dense clusters within the carrier (3B), and how the gelatin forms sheets that converge to form dense, supporting cords (3D). Another view of the gelatin-nylon carrier is seen in cross-section in figure 3C, showing the chambers formed by the sheets of gelatin, and the interaction between the gelatin (G) and the nylon fibers (N). It may be noted in 3C that the gelatin isn't simply precipitated on the nylon, but rather uses the nylon as anchor points from which the walls of chambers extend outward.

Evaluation of Bioreactor Performance.

Immuno-chemical evaluation of cell maintenance: An ELISA was used to determine the production of the liver-specific protein, serum albumin. The results of the ELISA confirmed what had been suggested by the morphology of the cells, namely, that viability and adult phenotype were maintained for extended periods. Figure 4 shows the RSA production over a 19 day period for cultures loaded with approximately $1x10^6$ cells/cm^2, and a 15 day period for cultures loaded at a density approaching $1x10^8$ cells/cm^2. Albumin production in both sets of cultures dropped sharply over the course of the first several days, but production levels began to rise at about day 7. The recovery was quite dramatic for the $1x10^6$ cells/cm^2 cultures, and RSA production nearly reached those seen for day 2.

Figure 3. Composite figures showing the structure of the cultures. 3A, Schematic representing one sheet of the cell carrier loaded with cells. 3B, Transverse view of a 3-week culture illustrating the dense arrangement of cells, and the cuboidal morphology typical of differentiated hepatocytes (Cc -- cell cluster, N -- nylon fiber); magnification = 660X. 3C, Cross section of a cell carrier illustrating the chambered architecture of the microporous gelatin sponge (G -- gelatin, N -- nylon fiber); magnification = 440X. 3D, Transverse view of a 3-week cell free control carrier illustrating the sheets (S) of gelatin that are visible with phase contrast microscopy, and how they join to form cords (C); magnification = 660X. Figures B and D are the same as 1A and 1B, respectively, and are shown here for reference.

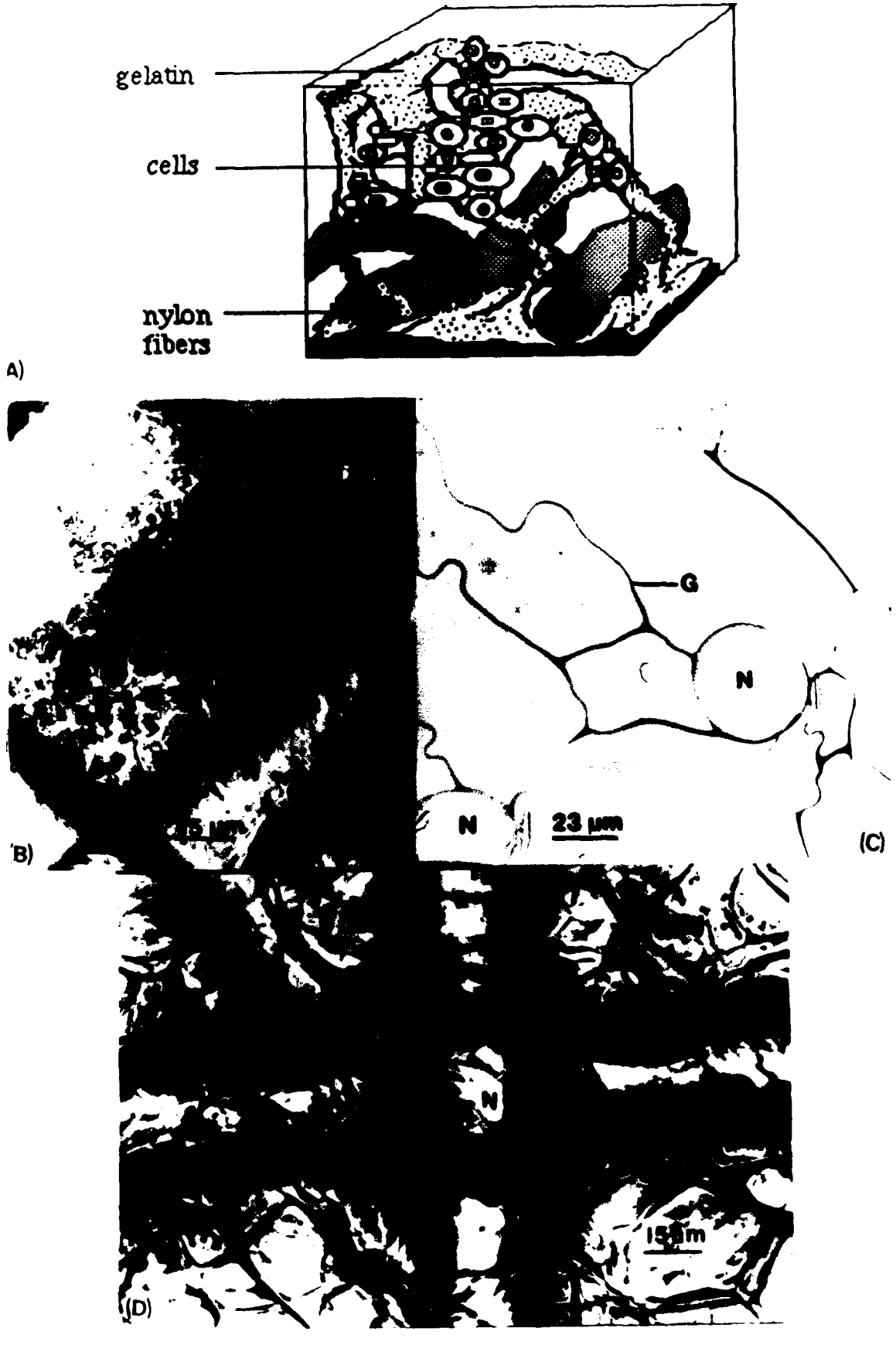
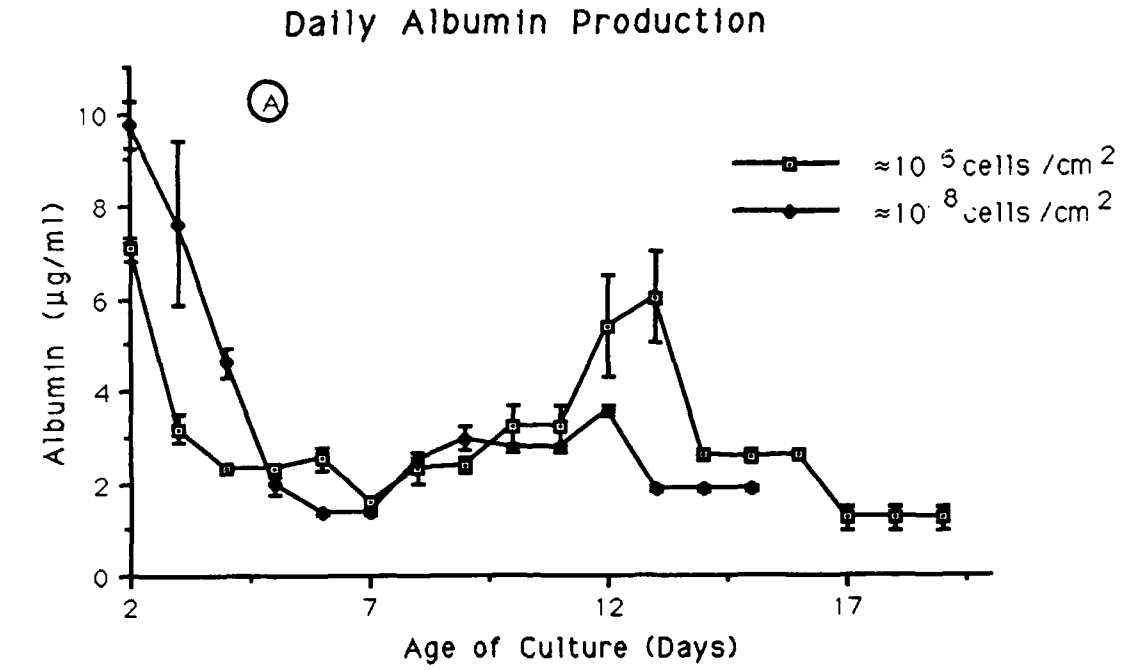
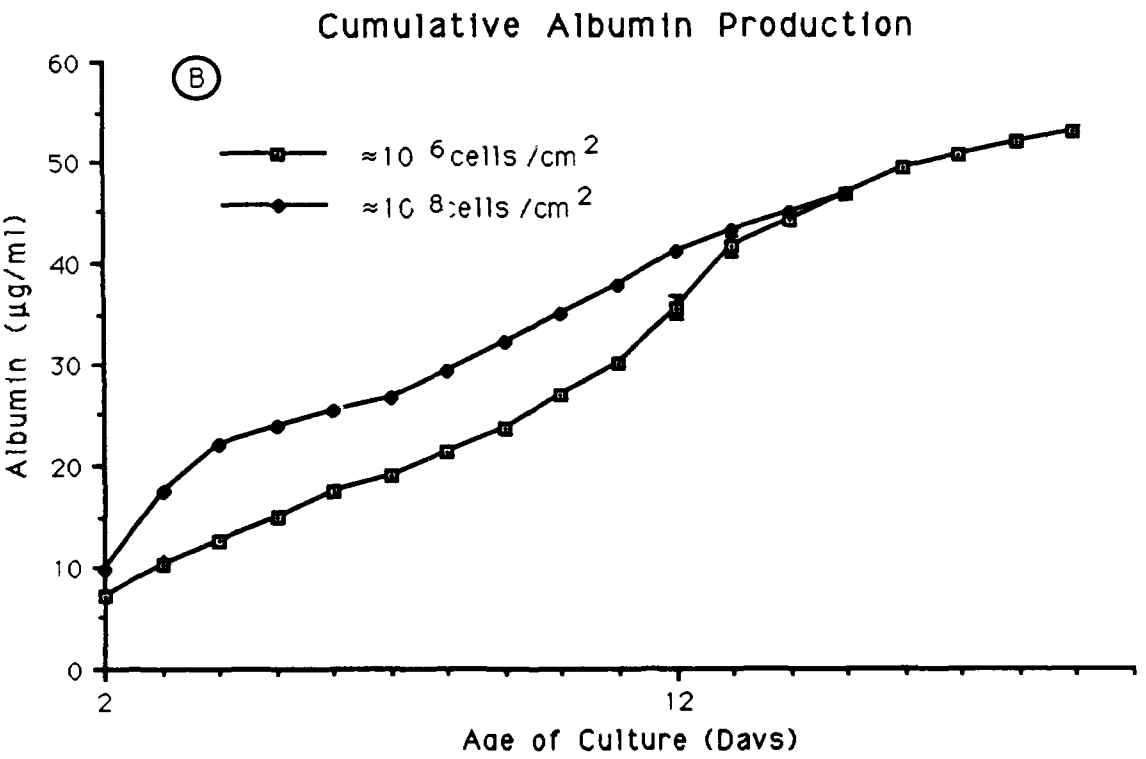

gelatin
cells
nylon fibers
A)
B)
G
N
N
23 µm
(C)
N
(D)

Figure 4. Albumin production by immobilized rat liver cells. Figure A represents daily production by the cultures, and figure B represents the cumulative albumin production over the course of the experiment. Cultures loaded at $\approx 1 \times 10^6$ were assayed for a 19 day period, while cultures loaded at $\approx 1 \times 10^8$ were assayed for 15 days; medium was changed daily.


Daily Albumin Production
A
≈10 5 cells /cm 2
≈10 8 cells /cm 2
Albumin (µg/ml)
0
2
4
6
8
10
2
7
12
17
Age of Culture (Days)


Cumulative Albumin Production
B
≈10 6 cells /cm 2
≈10 8 cells /cm 2
Albumin (µg/ml)
0
10
20
30
40
50
60
2
12
Age of Culture (Days)

After day 13 the production crested and started to drop again, but this time reaching lower levels than the first drop. It's questionable whether or not this wave-form pattern of albumin production would have continued had the cultures been allowed to go for longer periods of time. The recovery in the high density culture was surprisingly much lower, indicating either a loss of adult phenotype, and/or a possible shortage of nutrients. Because of this apparently reduced vitality in the high density cultures, all subsequent experiments were conducted using the $1x10^6$ cells/cm^2 cultures. The cumulative totals for the two sets of cultures are shown in figure 4B (note that the magnitude of the horizontal scale is much greater than in 4A). The apparent rapid decline in the vitality of the high density culture is evident in the shallow slope of the line after just a few days in culture (slope of the best fit line for the $1x10^6$ cells/cm^2 culture = 2.94, the slope of the best fit line for the $1x10^8$ cells/cm^2 = 2.60).

Production and Properties of Extracellular Immunogenic Vesicles From Liver Cells.

The objectives of this series of experiments were two-fold; 1) to see if a primary mixed liver cell culture could perform complex, multi-step *in vivo* functions *in vitro,* and 2) to determine what form exocytosed, processed antigen was presented in. Results of this work revealed the ability of a mixed-cell liver culture to endocytose supramolecular antigen and to re-express processed antigen in two forms -- large vesicles 4 and 5 days after inoculation, and another smaller form continuing over the course of 12 days at least.

Antigen uptake: The ability of the liver to take up antigen *in vivo* has been demonstrated repeatedly [20, 21], but whether disaggregated liver cells would be capable of endocytic uptake of antigen *in vitro* has not been investigated until now. Quantitation of free phage remaining in the media after 24 hrs showed that liver cell cultures removed significant quantities of antigen by endocytosis and by nonspecific adsorption to the carrier.

Figure 5 shows the percent of bacteriophage in the culture which remained free under various conditions after 24 hours, table 3 summarizes the results of the students t-test that was applied to the data to determine statistical significance between groups [56] and

Figure 5. Antigen uptake and non-specific loss at the end of 24 hrs of culture. Error bars represent standard error of the mean. Bars above the dashed line represent cell loaded roller bottles, while those below the line represent cell free bottles. All bottles contained L-15A medium and T2 bacteriophage. A (+) sign along the vertical axis indicates the bottle contained one or more of the following: Ca -- cell carrier, LPS -- lipopolysaccharide-B, CS -- 10% calf serum.

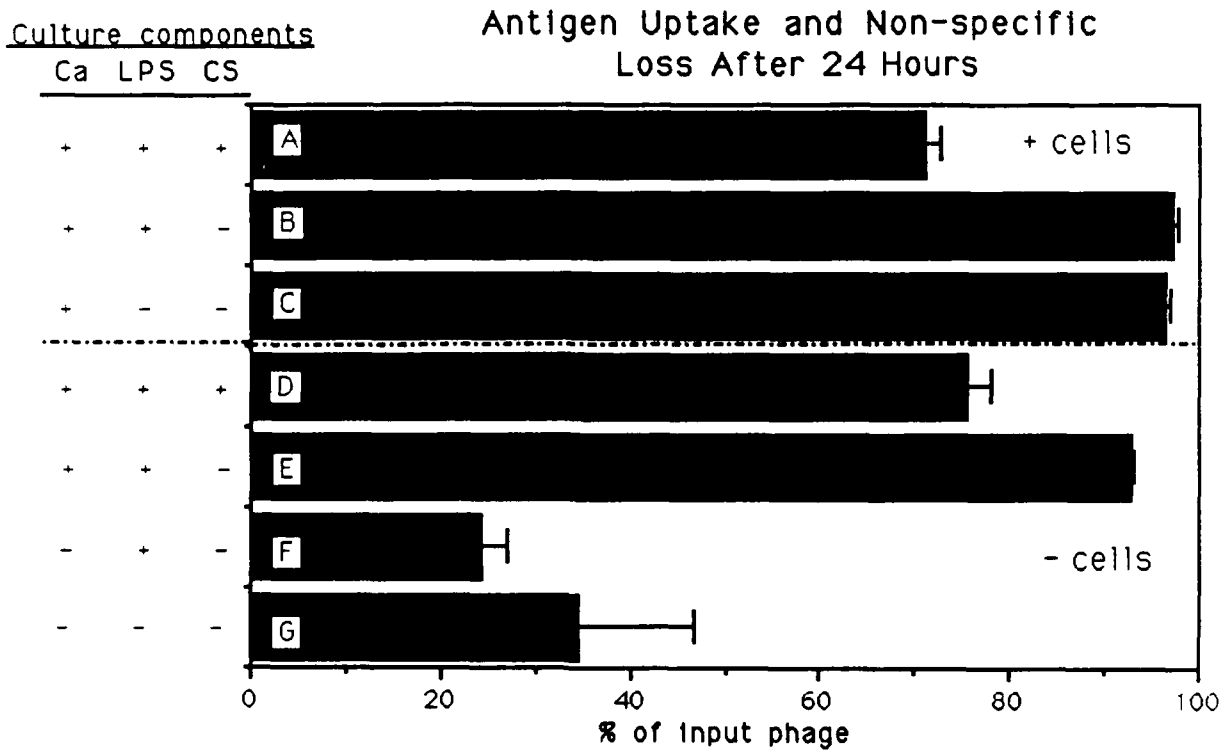

Antigen Uptake and Non-specific Loss After 24 Hours
Culture components
Ca LPS CS
+ + +
+ + -
+ - -
+ + +
+ + -
- + -
- - -
A
B
C
D
E
F
G
+ cells
- cells
0
20
40
60
80
100
% of input phage

Table 3. Statistical significance of antigen uptake assays.

	- Cells				+ Cells		
	medium only	LPS	Ca & LPS	Ca, LPS & CS	Ca & medium only	Ca & LPS	Ca, LPS & CS
Basal medium	--						
LPS	No	--					
Ca & LPS	Yes	Yes	--				
Ca, LPS & CS	Yes	Yes	Yes	--			
Ca	Yes	Yes	Yes	Yes	--		
Ca & LPS	Yes	Yes	Yes	Yes	No	--	
Ca, LPS & CS	Yes	Yes	Yes	No	Yes	Yes	--

Statistical significance in difference between groups was determined using the students t-test, $p = 0.05$. Yes indicates there is statistically significant differences between the groups; No means that there is no significant difference between the groups. Ca - Cell Carrier, LPS - lipopolysaccharide-B, CS - 10% fetal bovine serum. Groups listed vertically on the left side of the table which are above the line are cell free controls; groups below the line are cell loaded. Likewise, groups listed horizontally across the top which fall under "- cells" are cell free controls, and groups under "+ cells" are cell loaded.

table 4 is a summary of the conclusions made from this set of experiments. The cell-loaded, serum-free cultures (bars B and C, figure 5) appeared to take up ≈96% of the phage, as opposed to the cell free control (bar E, figure 5) which had an apparent uptake of ≈92%. Statistical analysis confirms that there was an active, cell mediated removal of phage from the media. It is interesting to note, though, that LPS, which is an immune stimulant, and/or adjuvant *in vivo*, had no demonstrable effect on the up-take of antigen by the liver cells *in vitro*.

To determine the causes of the apparent loss of free phage in the cell-free control, several other controls were incorporated into the experiment. The first control was a culture bottle that contained phage and medium only (bar G, figure 5) to see if non-specific adsorption of phage to the roller bottles was occurring, and/or if the medium was attenuating the virulence of the phage. Figure 5 shows that there was a minor reduction in the number of free phage able to infect host bacterium after incubation in the culture medium (≈25% apparent loss of free phage). The second control was a culture bottle that contained phage and LPS supplemented medium (bar F, figure 5) to see if the phage were binding to the LPS. Lipopolysaccharides in the outer wall of gram-negative bacterium are the natural receptors for coli-phage [17], so the possibility existed that phage were binding to the LPS and undergoing abortive expulsion of their DNA. However, the results show that binding of phage to LPS did not appear to occur; in fact, there was no significant difference between LPS supplemented bottles and media-only bottles (tables 3 and 4). This finding is not totally unexpected, since the source of the LPS added to the medium (*S. typhosa*) is quite different from the phage's host bacterium (*E. coli*). The third control bottle contained the cell carrier, phage and medium supplemented with LPS and 10% calf serum (bar D, figure 5) to see if reactive aldehyde groups on the cell carrier were binding the phage. Results indicate that about 15% of the loss of free phage may be attributable to non-specific binding of phage to free aldehyde groups (Figure 5). The final control was a cell-loaded culture supplemented with LPS and 10% calf serum (bar A, figure 5) to

Table 4. Conclusions from antigen uptake assays (figure 5).

No.	Treatment Comparisons	Conclusions
1.	A to B & A to D	Serum negates any apparent uptake of antigen by the liver cells; either due to competition for non-specific binding sites, or due to deleterious effects of serum on differentiated cells [19].
2.	B to C	LPS has no significant effect on the uptake of exogenous antigen by liver cells *in vitro*.
3.	B to E & C to E	There is a cell-mediated loss of free phage from the media -- liver cells are capable of endocytosing exogenous antigen *in vitro*.
4.	D to E	Serum appears to suppress the non-specific binding of phage to the carrier (by ≈15%). This indicates that phage probably are binding to free aldehyde groups on the surface of non-serum treated carriers.
5.	D to F	Approximately 50% of the apparent loss of free phage is due to entrapment of phage in the cell carrier's chambers.
6.	F to G	LPS has no effect on the apparent loss of free phage. Approximately 25% of the apparent loss of free phage is due non-specific binding of phage to the roller bottles' walls, and/or mitigating factors in the medium.

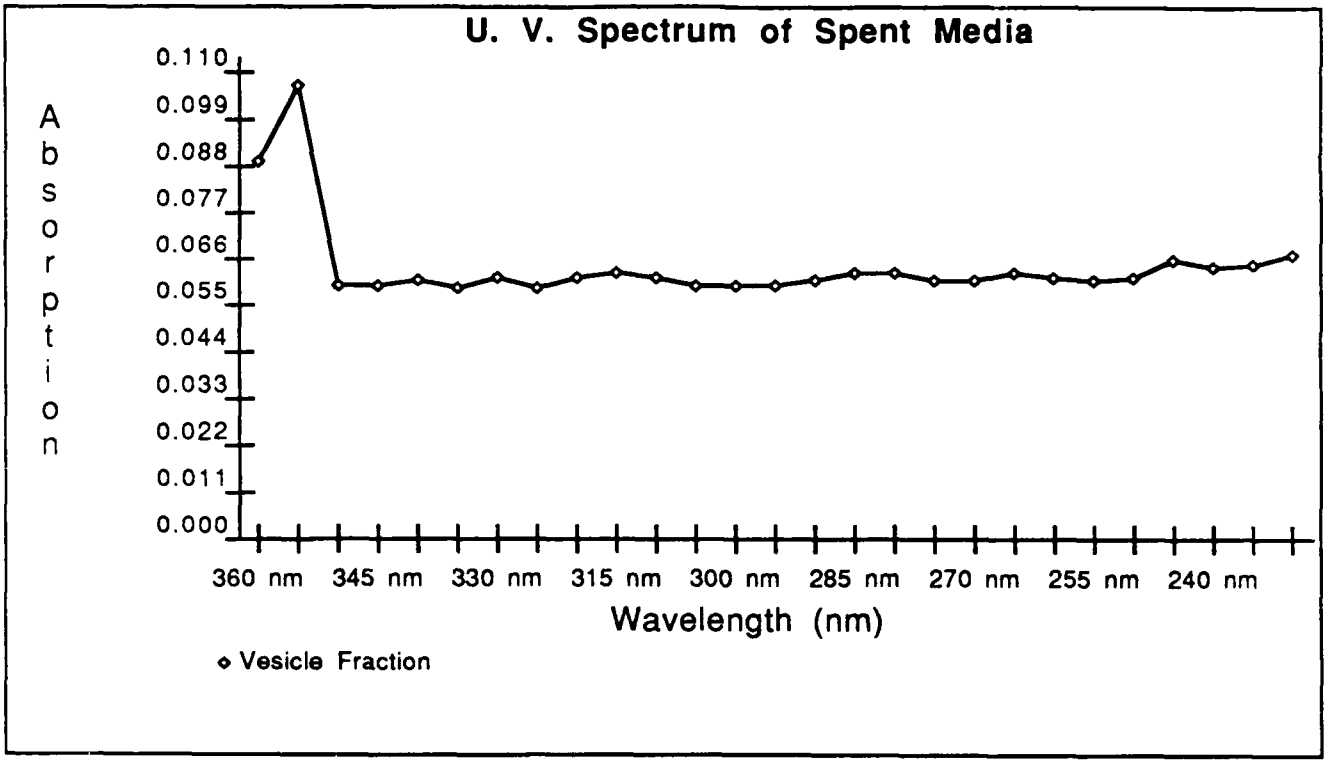
determine if the calf serum could block the free aldehyde groups without disrupting cell mediated uptake of phage. Figure 5 shows that the presence of serum appeared to eliminate the uptake of phage by the cells. This effect may have been due either to serum proteins out-competing phage for non-specific binding sites on the cells, or loss of endocytic ability by the cells due to serum factors.

In conclusion, ≈25% of the phage were lost due to non-specific binding to the roller bottle and mitigating factors in the medium, ≈50% of the phage were lost due to retention of phage in chambers of the microporous sponge, ≈15% of the phage were lost due to non-specific binding of phage to free aldehyde groups on the carrier, and ≈5% of the phage were actively endocytosed by the cells.

UV analysis of vesicles: Spent media was fractionated and a UV analysis was performed on the vesicles to see if they contained nucleic acid and protein (absorption peaks at 260 nm and 280 nm respectively). Presence of nucleic acid and protein together would indicate that the processed antigen in the vesicles resembled the RNPs previously reported [22, 24, 25]. The results of the UV analysis are given in figure 6. The strong absorption at 355 nm, and the relatively straight line through the rest of the spectrum suggest that the vesicles were simply scattering the light at most of the wavelengths, and that the only real absorption was probably due to membrane components (e.g. glycolipids). If there was any nucleic acid and/or protein expressed on the surface, it must have been a small quantity, small in size, or blocked by other components.

Electron microscopic examination of vesicles: Electron microscopy of the vesicle pellets provided the first information about (a) recovery of the liver cells from the stress of mechanical disaggregation, (b) morphology of vesicles shed naturally by healthy cells, and (c) evidence that antigen was re-expressed in vesicular form. Presence of antigen in the vesicles was determined using thin sections of embedded vesicles. Uranyl acetate staining of whole vesicles revealed their size and some of their physical characteristics.

Figure 6. UV spectrum of vesicles purified by fractionation of spent media on a Spectra/Gel A6 column.


U. V. Spectrum of Spent Media
Absorption
0.110
0.099
0.088
0.077
0.066
0.055
0.044
0.033
0.022
0.011
0.000
360 nm
345 nm
330 nm
315 nm
300 nm
285 nm
270 nm
255 nm
240 nm
Wavelength (nm)
Vesicle Fraction

Non-immunolabelled, ultra-thin sections: Ultrastructural examination of vesicle pellets from the first few days' spent media revealed two types of vesiculation by the cells. Figure 7A is a cross-section of the vesicle pellet from a 2 day old culture, showing a heterogeneous collection of structures. Most of the structures are very similar in appearance to cell organelles; such as the elongated, membranous structure covered with dense granular particles on the left of the figure, which is probably rough endoplasmic reticulum (rer); and the dense spherical structures on the right of the figure, which are probably lipid-bodies or lysosomes (L). The large, granular, spherical structures (diameters >0.5 μm) seen throughout the figure (B) fit the standard definition of "blebs". Figure 7B is a cross-section of a much more homogeneous pellet of membrane enclosed, spherical vesicles (diameters < 0.3 μm) from a culture which is 3 days old. In this pellet, which had been prepared for visualization exactly as the pellet in 7A, there is a distinct absence of any organelle-like structures. The presence of organelle-like structures in day 2 pellets, their absence from day 3 pellets, and knowledge that the cells were exposed to low temperatures (≈4° C for about 2 hours during the disaggregation and harvesting procedures) suggests that the blebs in figure 7A are not normal products of antigen processing and vesiculation, but the result of trauma experienced by the cells during their collection and isolation. Other researchers [65] have shown that treatments such as low temperature, colchicine and vinblastine, which damage cytoplasmic microtubules, result in formation of blebs very similar in size and appearance to those seen here. However, natural vesiculation of liver cells after 48 hours in culture is indicated by the absence of cell organelles in the day 3 vesicle pellet, and the distinctly different morphology of the day 3 vesicles from the blebs seen in the day 2 pellet.

Negatively stained, whole vesicles: Analysis of uranyl acetate stained vesicles by electron microscopy reveals several morphological features (figure 8A). Confirmed by this technique is the vesicles' roughly spherical shape; which had been suggested in cross sections of vesicle pellets (figure 7B). Very little staining is seen throughout most of the

Figure 7. Comparison of the vesicle pellet from a two day old culture, and from a three day old culture. 7A, Pellet from a two day old culture showing mixed cell fragments and vesicles; rer --rough endoplasmic reticulum, L -- lipid bodies, B -- blebs, magnification = 20,400X. 7B, Vesicle pellet from a 3 day old culture (magnification = 56,000X).

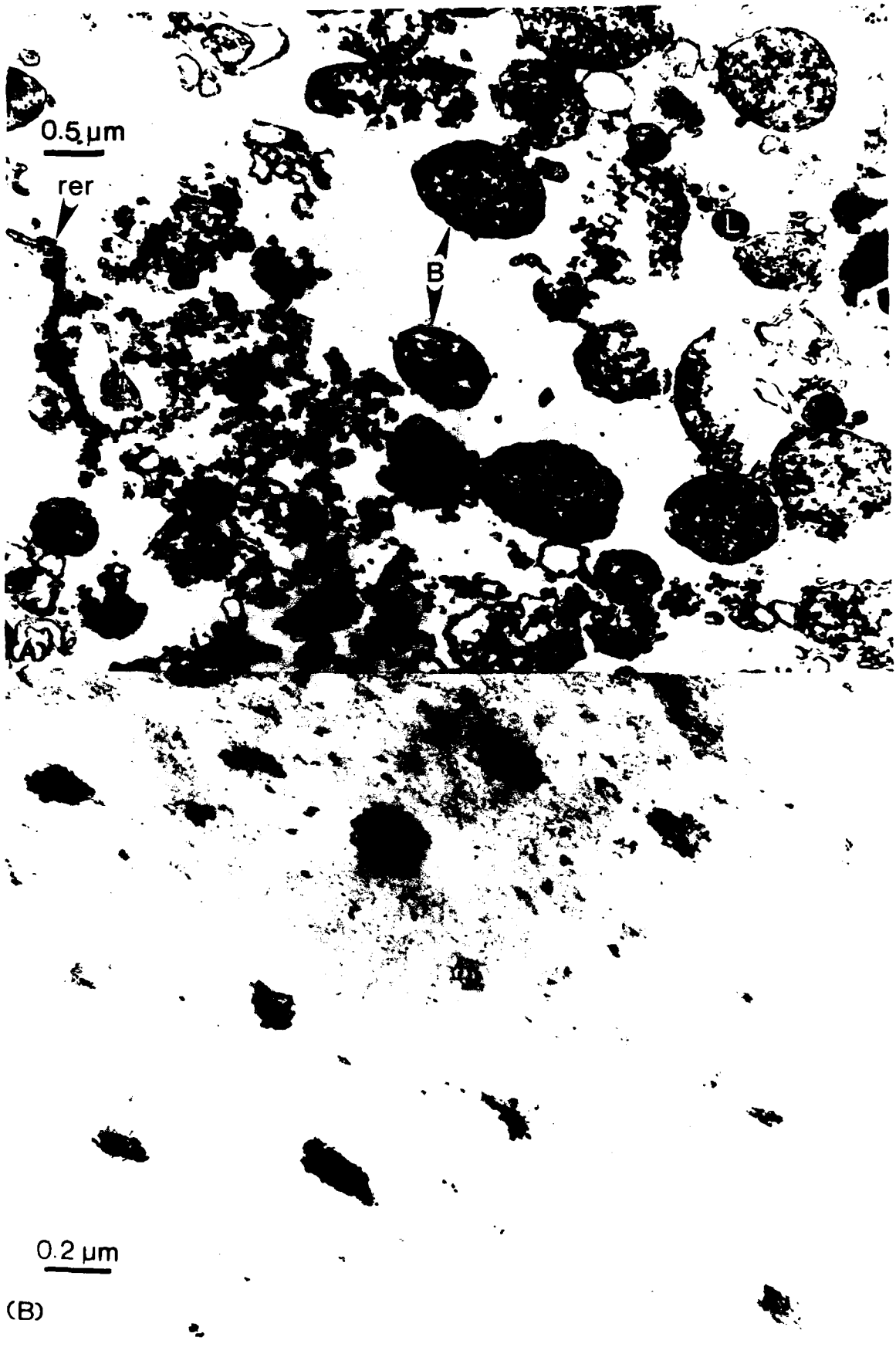
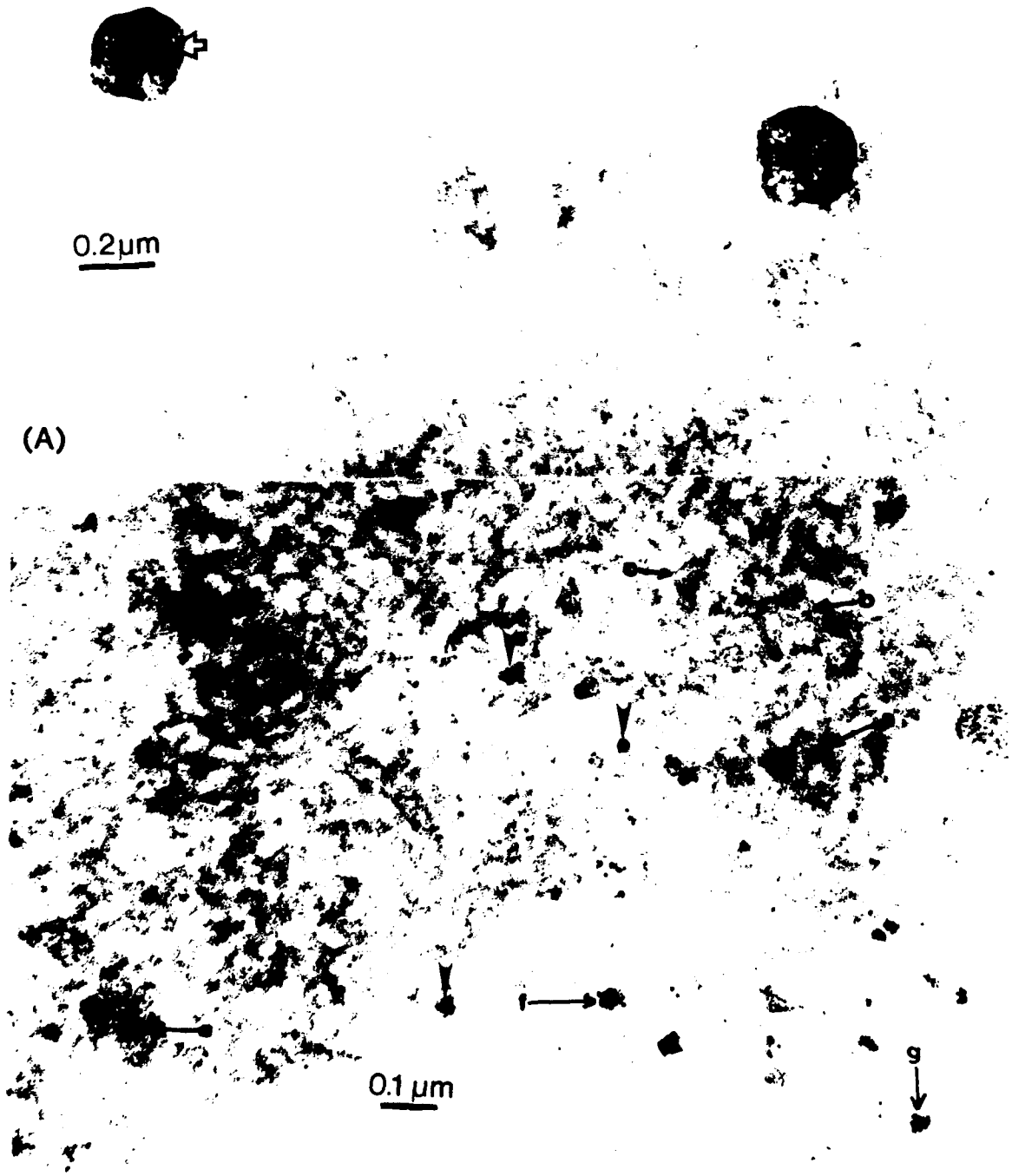

0.5 µm
rer
B
L
0.2 µm
(B)

Figure 8. Uranyl acetate stained whole vesicles, and immunolabelled vesicles from a KLH inoculated culture. 8A, Electron micrograph of whole vesicles stained with 0.5% uranyl acetate in PBS. Note the electron dense core in the vesicle on the left (arrow). Magnification = 56,000. 8B, Cross section of agar-embedded vesicles immunolabelled for KLH (5 nm gold particles) and MHC I (10 nm gold particles). The letters and arrows indicate labelled structures suggestive of the cores seen in figure 8(A); the order of lettering was a matter of convenience and has no other significance. Arrow heads indicate what are presumed to be lead citrate precipitates. The high background staining is due to the agar used as a vesicle support. Magnification = 84,000X.


0.2µm
(A)
0.1 µm
g

vesicle. This supports the idea that the vesicles are composed of a bilipid membrane (non-osmicated bilipid membranes are known to stain lightly with uranyl acetate [12]). Another feature which is evident from analysis of the uranyl acetate stained, whole vesicles (figure 8A and others not shown) is the vesicles' size; their diameter is approximately 300 nm. On close inspection a dense, kidney-shaped core within the vesicles can be seen (arrow). One can hypothesize that the core is the RNP antigen described by others [22, 24, 25]. Support for this comes from the fact that RNPs stain densely with uranyl acetate [12], and from the following observations made with immunolabelled vesicles.

Immunolabelled ultra-thin sections of vesicles: Antigenic fragments of the KLH used to inoculate mixed liver cell cultures have been confirmed present in the vesicles through the use of gold conjugated-antibodies. Figure 8B is an electron micrograph of a cross-section made from day 4 vesicles suspended in agar and assayed for the presence of KLH (5nm gold particles) and MHC-I (10 nm gold particles). The immunolabelled, antigenic fragments in the figure (indicated by the letters a - g) are KLH fragments. Based on negative controls, at least 64% of the KLH labels are due to specific anti-KLH - KLH reactions (figure 9A). In this figure there are no clear indications of the presence of MHC-I. The large, irregular inclusions are presumed to be lead citrate precipitates and not the 10 nm gold particles used for MHC labelling (arrow heads). The latter are indicated with a solid arrow in figure 9B. Bleaching the osmium tetroxide from the sectioned vesicles prior to immunolabelling made subsequent contrasting of membranes with uranyl acetate and lead citrate difficult. Nevertheless, the immunolabelled structures' slightly darker staining helps in their visualization. There is an interesting similarity between some of the immunolabelled figures in 8B (particularly structures b, c and d), and the dense core (open arrow) of the vesicle in figure 8A. The similarity of the immunolabelled structures in 8B to the dense staining core in 8A lends tantalizing support to the hypothesis that the cores within the vesicles are the actual carrier of the antigen.

Figure 9. Immunolabelling controls for KLH and MHC-I.

9A. Electron micrograph of negative control for the immunolabelling of KLH (5 nm gold particles)and MHC I (10 nm gold particles). Two of the 5 nm labels are indicated by arrow heads; there was no background adsorption by the 10 nm labels. Magnification = 84,000X. 9B, Positive control labelled for both KLH (hollow arrow) and MHC I (solid arrow); magnification = 84,000X. 9C, Section of the micrograph seen in figure 8B, and is provided here for comparison (arrow heads indicate KLH containing structures; magnification = 84,000X.


1 µm
0.1 µm
(B)
(C)

Unfortunately, the double labelling experiments failed to either confirm or deny the presence of MHC-I and MHC-II antigens on the vesicle surfaces. It can only be said that MHC-I was being shed by the cells (figure 9B) and it subsequently pelleted with the antigen-containing vesicles.

Quantitation of exocytosed antigen: Immunochemical determination of the amount of antigen exocytosed by the cells not only confirmed that specific antigen was contained in the vesicles (as had been demonstrated by the immunolabelling experiments), but also indicated that a second species of processed antigen was being actively exocytosed by the cells. It had been expected that KLH from the initial inoculation would be diluted out with daily media exchanges, and a peak of antigen exocytosis would appear in the vesicle fraction between days 3 and 5. As anticipated, there was a small carry-over of raw antigen for about a week, and antigen in the vesicle fraction did appear at days 4 and 5 (figure 10B). The amount of antigen exocytosed in vesicular form was ≈ 0.6 pg/cell in the LPS-free cultures; no antigen was exocytosed in vesicular form by the LPS-supplemented cultures. A quite unexpectedly high concentration of processed KLH was found in the supernatants which persisted for the entire 12 days of the experiment (figure 10A). A total of ≈ 8.4 pg/cell of this form of secondary antigen was exocytosed by the LPS-supplemented cultures from day 3 to day 12; only ≈ 5.0 pg/cell of this form of antigen was exocytosed by the non-LPS supplemented cultures for the same period. It is interesting to note that the addition of LPS to the cultures had a negative effect on the release of antigen in vesicular form, but a positive effect on the exocytosis of the smaller form of antigen. LPS-supplemented cultures exocytosed almost twice as much of the smaller antigen as the non-LPS-supplemented cultures. Figure 11 re-expresses the data as percentages of the KLH used to inoculate the cultures. This was done simply to get a better appreciation for the efficiency of the system.

Figure 10. Concentration of processed antigen exocytosed into the spent medium. 10A, Processed antigen which remains in the supernatant after 70,000Xg centrifugation. CK cultures inoculated with KLH only; CLK cultures inoculated with both KLH and LPS. 10B (insert) Vesicular processed antigen exocytosed by non-LPS supplemented cultures inoculated (CK).

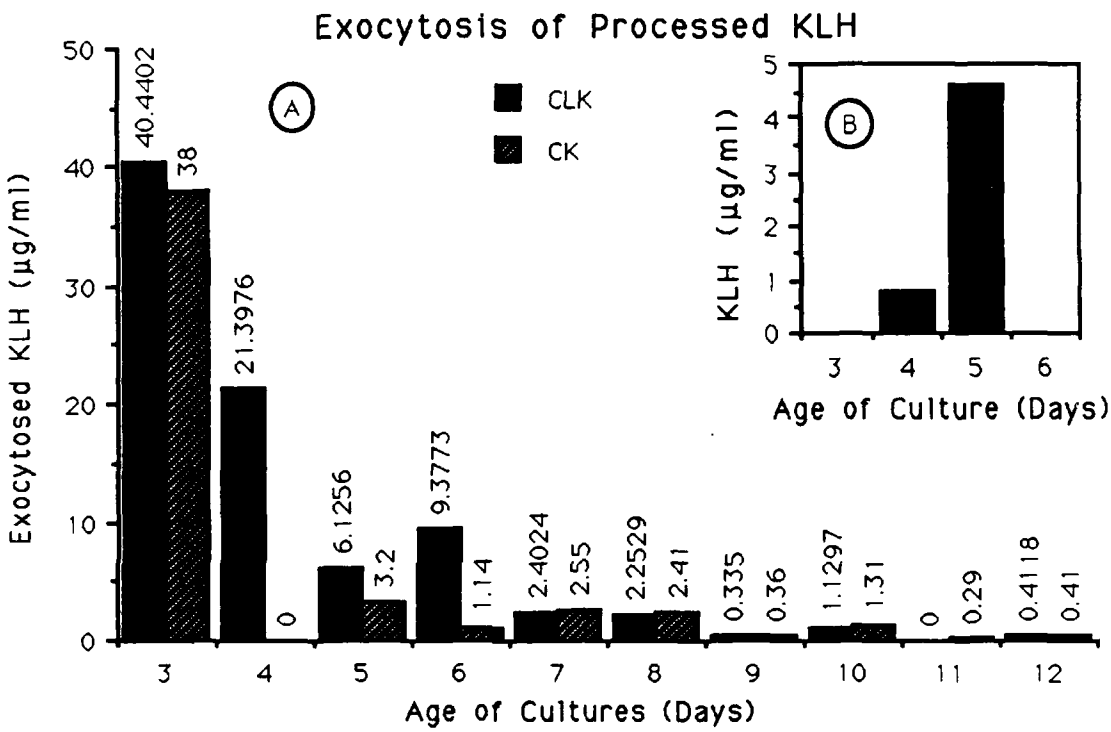
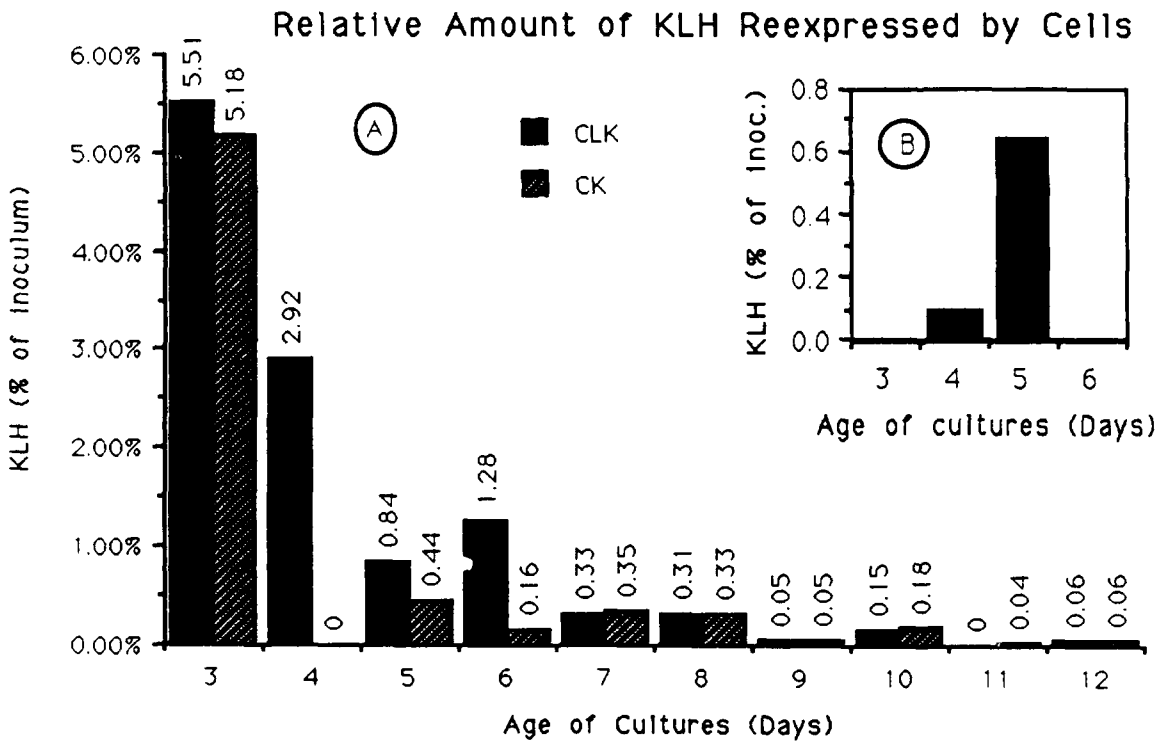

Exocytosis of Processed KLH
Exocytosed KLH (µg/ml)
50
40
30
20
10
0
A
CLK
CK
40.4402
38
21.3976
0
6.1256
3.2
9.3773
1.14
2.4024
2.55
2.2529
2.41
0.335
0.36
1.1297
1.31
0
0.29
0.4118
0.41
3
4
5
6
7
8
9
10
11
12
Age of Cultures (Days)
KLH (µg/ml)
5
4
3
2
1
0
B
3
4
5
6
Age of Culture (Days)

Figure 11. Percent of KLH inoculum returned to the spent media in processed form. 11A, Small species of processed antigen (CK represents cultures inoculated only with KLH; CLK represents cultures inoculated with KLH and LPS). 11B, Large vesicular antigen from non-LPS supplemented cultures.


Relative Amount of KLH Reexpressed by Cells
KLH (% of Inoculum)
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
5.51
5.18
2.92
0
0.84
0.44
1.28
0.16
0.33
0.35
0.31
0.33
0.05
0.05
0.15
0.18
0
0.04
0.06
0.06
3
4
5
6
7
8
9
10
11
12
Age of Cultures (Days)
A
CLK
CK
B
KLH (% of Inoc.)
0.8
0.6
0.4
0.2
0.0
3
4
5
6
Age of cultures (Days)

DISCUSSION

The experiments reported here demonstrate that it is feasible to use primary mixed liver cell cultures to produce extracellular, vesicular antigens. A microporous cell culture system was developed which is inexpensive to produce, adaptable to various bio-reactor designs and able to support differentiated liver cells for prolonged periods of time in minimal medium. It is a first step toward the goal of designing a reactor for high yields of processed-antigen. A significant second achievement was proof that liver cells can endocytose exogenous antigen *in vitro* and exocytose it in a vesicular form.

The gelatin-based cell carrier developed for this work has several advantages over many previously described cell substrates in which collagen has been precipitated onto nylon mesh or other mechanical carrier. This system generates an adsorptive surface (gelatin) with an open foam, and not a glossy or flocculent precipitate [13, 58]. Collagen is both expensive and unstable. The derivatized gelatin, however, is stable at room temperature and is inexpensive. This stability allows cell carriers to be produced ahead of time and stored compactly. One special advantage of the nylon-gelatin cell carrier is the way in which cells become established in the cavities of the microporous gelatin sponge, essentially encapsulating the cells. The advantages of encapsulated cells have been reviewed recently in other studies with rat hepatocytes [4]. One great advantage that is generally recognized is the increased ratio of culture surface to the medium. Many other reactor configurations have been devised to increase cell culture surface, including roller bottle cultures, microcarriers, and glass fiber bed reactors [52]. For our work the roller bottle was chosen for the relatively high ratio of cells to medium which can be maintained, and because it permits maximal diffusion of oxygen to the cells at ambient pressure. The importance of oxygen availability to hepatocytes had been demonstrated previously, using floating collagen gels. These supported cells for much longer periods of time than cultures anchored to the bottom of petri plates [47].

The use of a mechanically disaggregated, mixed cell population has been exceedingly successful in maintaining the cells' phenotype and viability in a defined medium. This was achieved in spite of the conventional wisdom that such cells would be irretrievably damaged. The fact that the tissue was not completely disaggregated to a population of single cells (as mentioned previously, cell aggregates ranged from 1 to ≈ 15 cells) certainly was a reason for the greater success rate seen here. Using albumin production and morphological examinations as indicators of cell health, it was demonstrated that hepatocytes, at least, maintained a differentiated phenotype for a minimum of 19 days. Furthermore, light microscopic examination of the cultures showed that intercellular contacts were regenerated between cells, to the point that the morphology of the cell aggregates were reminiscent of the *in vivo* morphology of the liver (figure 2). It may be concluded that coarse disaggregation and the avoidance of proteolytic enzymes contributed to formation of intercellular contacts and preservation of the surface molecules hypothesized to be the communicators between epithelial and parenchymal cells.

Support for this conclusion comes from the extensive co-culture studies done by Guguen-Guillouzo's and Mesnil's groups [26, 46]. They also found that albumin production by hepatocytes in co-culture with other liver cell types was typified by alternating waves of decreasing intensity (figure 4). Their reports have shown that while intercellular contact between parenchymal and epithelial cells made a significant contribution to differentiation in hepatocytes, the hepatocytes and epithelial cells did not actively pass material from one cell to another (as determined through the use of fluorescent dyes). Rather it appears that the two different cell types communicate through surface molecules such as adhesins, glycoproteins, glycolipids, etcetera. It has been suggested, though, that gap-junctional communication does exist between hepatocytes in culture, since it has been found that hepatocytes which are not in direct contact with epithelial cells are still influenced by their presence in the culture [46]. The findings in this thesis are further supported by reports that matrices of basement membrane proteins on all sides of the

parenchymal cells enhance and maintain differentiation [5, 13]. Thus it appears that preservation of intercellular contacts between cells, and the extracellular matrix around them during cell harvesting is more effective than trying to regenerate the membrane structures after complete, proteolytic disaggregation.

It is well documented that the liver is a site of uptake and proteolysis of foreign proteins *in vivo* [20, 22, 28], but the phagocytosis of macromolecular and supramolecular structures by liver cells *in vitro* has received very little attention. The general mechanisms of endocytosis by *in vivo* liver cells has been reviewed elsewhere [70], and will be discussed here only briefly. In the *in vivo* liver, large foreign particles are first marked by immunospecific.(e.g. opsonins) and nonimmunospecific (e.g. fibronectins) recognition factors in the circulation. The marked particles are then phagocytosed by Kupffer cells via activated membrane receptors. Hepatocytes have been found to take up small particles via fluid-phase endocytosis, a constitutive process by which cultured hepatocytes have been found to endocytose up to 20% of their cell volume [55].

This work indicates that a mixed liver cell culture is capable of exogenous antigen uptake in the first 24 hrs of culture, perhaps using the same mechanism as that used by liver cells *in vivo*. In the case of T2 bacteriophage, the cells are able to take up about 4% to 5% of the T-2 used to inoculate the cultures, which equates to about 200 - 250 phage per cell. Considering the large size of the T2 bacteriophage (approximately 100 x 250 nm [17]) and the fact that there are no T2 receptors on liver cells, it seems likely that active uptake of T2 is initially accomplished through phagocytosis by non-parenchymal cells. This hypothesis is supported by the demonstrated ability of hepatocytes in the mixed cell culture to produce the serum-protein albumin; which indicates that they are also capable of producing other serum factors (i.e. recognition factors) required for Kupffer (and probably endothelial) cell mediated phagocytosis. As discussed in the introduction, endocytosis of large particles in the liver is thought to be accomplished primarily by non-parenchymal cells, which are activated by specific and non-specific recognition factors bound to the

particles [70], while hepatocytes are restricted to endocytosis of smaller particles. It is likely that the non-parenchymal cells release a partially degraded form of the antigen into the medium, where it may then be taken up by the hepatocytes for further processing. In fact, a similar method of "conveyor belt" antigen transport and processing has been investigated in the popliteal lymph nodes of rats [62].

Although the results of the UV analysis of the vesicles failed to either confirm or deny the presence of nucleic acid, it did yield some other potentially useful information. There is the strong absorption peak at 350 nm which suggests the presence of glycolipids. This suggests the future use of lectin columns for the separation and concentration of vesicles a potential option [44].

Results from other experiments suggest that several steps are required before UV analysis of the vesicles can yield information about the co-presence of nucleic acid and protein in the processed antigen. First, the vesicles must be collected in greater quantity to amplify the processed antigen -- electron micrography and immuno-chemical analysis has shown that only small amounts of antigen are present in the vesicles. Second, the presence of the lipid membrane, as confirmed by electron micrography, should be removed to eliminate its possible interference with internal components' absorption.

The physical characteristics of the vesicles as determined by transmission electron microscopy may provide important clues to antigen processing by liver cells, as well as how the vesicles may be employed by the immune system (see table 5). The presence of the antigen in a core structure which stains darkly with uranyl acetate lends support to the speculation that the antigen is in the form of a RNP. In non-osmicated tissues, uranyl acetate has been shown to stain RNPs strongly and plasma membranes moderately to weakly [12]. Vesicles in figure 8A have mostly translucent membranes, but the cores stand out boldly (note the vesicle on the left). These forms suggest that at least some of the RNP antigen produced by liver cells may be packaged in spherical, membrane enclosed vesicles. It is speculative but possible that the size of the vesicles offers some clues about their role

Table 5. Summary of processed antigen characteristics.

Characteristics	Large, Vesicular Antigen	Small Processed Antigen
Size (diameter)	300 ± 50 nm	unknown
membrane enclosed	yes	unknown
light absorption peak(s)	350 nm	unknown
precipitating g-force	≤22,000Xg	>70,000Xg
specific antigen carrier	yes	yes
time of exocytosis	days 4 &5	*by day 3 though day 12
effect of LPS	negative	positive
presumed immunological role	anamnestic response	antigen presentation in primary immune response

* The small processed antigen may be exocytosed as early as day 1, but the presence of free molecular KLH in the day 1 medium, and the cellular rupture occurring on day 2 make its identification dubious.

in the immune system. The antigen-containing iccosomes that have been studied by Szakal, et. al. [62] are essentially the same size as the vesicles that are shed by the liver cells. The iccosomes' range in size from 0.25 to 0.70 μm [63], while the vesicles from the liver cells have been estimated to range in size from 0.25 to 0.35 μm (based on results of this study). Szakal, et. al. have suggested that the size of the iccosomes contributes to the recognition and stimulation of B cells, as well as endocytosis by B cells and macrophages (phagocytosis of vesicle-associated antigens by macrophages has also been described in tumor cell systems [65]).

Several important points must be established before meaningful comparisons can be made between iccosomes and hepatic vesicles. Iccosomes are products of previous interactions between antigen and antibody, and so internalize the entire antigen-antibody complex [63]. They have also been shown to possess the MHCs necessary for presentation of their antigens and interaction with B cells [63]. Liver generated vesicles, on the other hand, are products of primary antigen response, and contain only antigen (since there were no antibody producing cells in the cell culture systems). Also, conclusive demonstration of MHCs on the liver-produced vesicles is still lacking. Still, it is quite probable that the packaging of processed antigen in vesicles would enhance specific endocytosis of the antigen by other APCs. This hypothesis would be a logical parallel to the findings that iccosomes and vesicles shed by tumors are phagocytosed by macrophages, which in turn present the antigen to T-cells [63, 65]. It would also complement the finding that *in vivo* processed antigen in the liver is released after secondary exposure to the antigen [20]. In this fashion, the vesicles could increase the apparent concentration of antigen in the system by avoiding reaction with free antibody and allowing expression of the antigen on the surface of APCs. The APCs in turn stimulate B cell growth, thus contributing to the heighted response typically seen in secondary exposures to antigen.

The assay of exocytosed antigen both confirmed previous hypotheses regarding presence of antigen in the vesicles and vesicle dynamics, and suggested new ones. The lone presence of antigen in day 4 and day 5 vesicles coincides with findings that the antigen-RNA complexes in hepatocytes leave the nucleus and migrate toward the plasma membrane by the third day after exposure to antigen [66]. This time frame for antigen processing in the mixed liver cell cultures also coincides with the iccosome system [62]. In that system, antigen containing iccosomes were dispersed between 3 and 5 days after inoculation. What was unexpected was the high concentration of antigenic material in the supernatant after the spent media had been centrifuged at 70,000Xg. Two explanations are possible. First, the material may represent either partially degraded antigen which had been released from the non-parenchymal cells but not yet endocytosed by the hepatocytes, or partially degraded antigen which is repeatedly cycled through the hepatocytes (it has been demonstrated that up to 80% of radiolabelled inulin endocytosed by *in situ* hepatocytes is immediately re-exocytosed into the plasma [55]). Alternatively the hepatocytes may release at least two species of processed antigen; one which is the vesicle enclosed species visualized with electron microscopy, the other a smaller and/or non-vesicular element. Neither alternative can be confirmed as yet.

It could be questioned further whether the two species of processed antigen have separate target cells in the organism. Secondary challenge of animals with complete antigen caused processed antigen from the first challenge to be released from the liver, and transferred to the spleen and lymph nodes (though predominantly to the spleen) [20]. If one applies the mechanism seen in the popliteal lymph nodes [62] to the spleen, then liver generated vesicles may be targeted for the germinal center lymphocytes. Thus it could play a role in the anamnestic response (i.e., immune memory and heightened response to subsequent antigen challenges). The smaller species might have as its target population circulating leukocytes and lymphocytes, and thus contribute to the initial immune response against new antigen. This model would help account for the high concentration of the

smaller antigen seen shortly after inoculation, and the relatively low concentration of vesicular antigen seen only on days 4 and 5.

This work started with the premise that it was necessary to activate the antigen processing machinery before antigen uptake could occur. In support of this premise, the *in vivo* immune stimulant LPS was assayed for its effects *in vitro*. It can be concluded from the results seen in table 4 that it has minimal or no effect on antigen uptake. From figures 10 and 11 (and table 5), it can be concluded tentatively that it inhibits the production of vesicular antigen, but stimulates the release of the smaller species of antigen. These results, though different from the enhancing effect of LPS *in vivo*, may be explained by the interaction between LPS and phospholipids on cell membranes [38]. This causes a cascade of effects (membrane activation), and some of the effects may include disruption of contacts between membrane proteins and microfilaments. Interference with the membrane-cytoskeletal networks seems a likely reason why antigen is not evident in vesicles from LPS supplemented cultures. In future work, where the vesicular form of the antigen is the preferred product, it may be better to avoid the use of LPS.

This research demonstrated that a mixed cell liver culture has the potential for producing novel vesicular antigens. However, there are related areas which should be explored in future work to refine the bioreactor and to shed more light on the antigen processing mechanism of liver cells. Varying the proportion liver cell types in the culture should have some effect on antigen processing by the bioreactor and may also affect the longevity of the culture. Maximizing the production of vesicular antigen is an obvious objective and may be attained simply by a second inoculation to stimulate release of processed antigen stored in the cells [20, 22]. Immuno-localization of surface antigens on the vesicles should refine characterization of the vesicles and gain insight into the mechanism of antigen processing. More efficient methods for the collection and purification of vesicles are needed. Characterization of the smaller species of antigen should be done in order to determine if it is a product of the liver cells with specific

modifications/ligands, or if it is simply a reduced form of the raw antigen. Finally, there should be a study of new supplements to the medium for increased culture longevity. Media additives which warrant consideration include: ascorbic acid which is an enhancer of collagen synthesis and has a half-life of only 12 hrs in culture [41]; and a combination of factors (amino acids, fatty acids, a protease inhibitor, vitamins and trace elements) found [48] to improve hepatocyte maintenance in culture.

CONCLUSION

This research has shown that primary mixed liver cell cultures offer the possibility of a novel vaccine source. An inexpensive and versatile cell carrier, combined with mechanical disaggregation of the tissue allows for the prolonged maintenance of a differentiated liver cell culture. Advantages of the carrier include its resistance to shear forces and the encapsulation of the cells, resulting in an increased culture surface area to medium ratio. In culture, the cells display the morphological features of differentiated cells and produce tissue specific proteins on a long term basis. Cross sections of the immobilized cultures suggest that the cells aggregate into cords reminiscent of the original liver tissue sinusoids. Two of the functions of whole liver which have been demonstrated in this cell system are antigen up-take and packaging of exogenous antigen. One of the forms in which this processed antigen occurs is a 250 - 350 nm vesicle, which envelopes a densely staining, antigen complexed core. The characteristics and dynamics of the vesicular antigen resemble at least superficially the antigen-RNA complexes described by Garvey and Reilly [22]. It is suggested that it may function in the immune system in a way similar to that described for iccosomes in lymph nodes [63]. In the course of studying the dynamics of vesicular antigen production, a second species of processed antigen has also been found which is exocytosed by the cells over the course of at least 12 days.

APPENDIX A: MEDIA, BUFFERS, AND REAGENTS.

All media and buffers were made using glass distilled water.

L-15A Medium, pH 7.4 (38).
L-15 medium prepared as per manufacturer's instructions (GIBCO, Grand Island, NY) fortified with;
3.57 g/l HEPES
50 U/ml penicillin (1 ml of GIBCO prep. / 100 ml of media)
50 U/ml Streptomycin (1 ml of GIBCO prep. / 100 ml of media)
250 mg/l Fungizone (1 ml of GIBCO prep. / 100 ml of media)
1.5 g/l glucose
0.5 mg/l insulin
2.2 g/l $NaHCO_3$
1.0 mg/l transferrin

HBSS, -Mg^{2+}, -Ca^{2+}, with EGTA (GIBCO, Grand Island, NY).
8.0 g/l NaCl
0.4 g/l KCl
0.06 g/l KH_2PO_4
0.05 g/l Na_2HPO_4
1.0 g/l Glucose
0.01 g/l Phenol Red
0.19 g/l EGTA.

PBS, 0.01 M, pH 7.2 (GIBCO, Grand Island, NY).

Solution A:	Solution B:
0.79 g of $NaH_2PO_4*H_2O$	2.04 g of Na_2HPO_4
4.76 g of NaCl	12.24 g of NaCl
dH_2O to 560 ml.	dH_2O to 1440 ml.

Autoclave the 2 solutions separately, allow them to cool, then mix to yield 2 l of PBS.

PBG Buffer. (Janssen Life Sciences Products, a division of Biotech N. V.).
Use the PBS buffer described above, and supplement with the following:
0.5% BSA
0.1% Gelatin (EIA grade)
20 mM NaN_3

0.1 M Carbonate-Bicarbonate Buffer, pH 9.6, with 0.01% Sodium Azide.
1.77 g of Sodium Carbonate (Na_2CO_3)
7.00 g of Sodium Bicarbonate ($NaHCO_3$)
0.1 g of Sodium Azide (NaN_3)
Dissolve in 1000 ml of dH_2O.

ELISA Washing Buffer
This buffer is the PBS buffer described above, with the following supplements
0.05% Tween 20 (500 µl / l)
0.05% Gelatin (EIA grade) (0.5 g / l)
0.01% Sodium Azide (NaN_3) (0.1 g / l)

ELISA Substrate Buffer, 0.01 M Carbonate-Bicarbonate Buffer, pH 10
Dilute the 0.1 M Carbonate-Bicarbonate Buffer 1:10, and adjust pH to 10.
1 mg/ml of p-nitrophenol phosphate, disodium (Sigma Chemical Co., St Louis, MO.)
1 µl/ml of 1 M $MgCl_2$ in 10 mM HCl (Sigma Chemical Co.)

NH_4Cl Buffer (for lysing red blood cells) (9).
2.06 g Tris base (0.017 M)
5.87 g NH_4Cl (0.1 M)
dH_2O to 1 liter, pH to 7.2.

Tryptone Broth (for the culture of *E. Coli* B) (Ref. Adams, M. H. 1959. The Bacteriophage, Interscience Publ. Inc., New York, NY, p. 445).
10 g Tryptone
5 g NaCl
5 g Yeast Extract
1 liter dH_2O

Biuret Reagent. (7).
1.5 g Cupric Sulfate $CuSO_4 * 5\ H_2O$
6.0 g Sodium Potassium Tartrate $NaKC_4H_4O_6 * 4\ H_2O$
Dissolve the above two reagents in 500 ml of dH_2O. To this solution, add 300 ml of 10% NaOH with thorough mixing. Dilute to 1 l. If precipitate forms, discard the reagent.

APPENDIX B: SURGICAL, IMMUNOLOGICAL, SEROLOGICAL TECHNIQUES.

Surgery Protocol: Details for excision of the liver from rats follows:

1). The animal was anesthetized with ether and its ventral side was shaved, and washed with 70% ethanol to provide a sterile working area.

2) A mid-line incision was made, and then diagonal incisions from the mid-line towards each of the legs was made so that large flaps of skin could be folded back from the abdomen. The peritoneum, muscle and fat layers were similarly cut, and all layers, including the skin, were held out away from the abdomen using forceps.

3) The intestines were reflected to the left of the animal to expose the right kidney, inferior vena cava and the lower portions of the liver.

4) Using curved hemostats, the vena cava was clamped between the renal artery and liver, and again between the liver and the diaphragm.

5) After the portal vein was cut, the lobes of the liver were excised and immediately transferred to a sterile petri plate containing HBSS, -Mg^{2+}, -Ca^{2+}, with EGTA (see appendix A).

NOTE: Animal care, anesthesia and sacrifice met the standards established by the Syracuse University Office of Laboratory Animal Research, and guidelines of US Public Health Service Policy on Humane Care and Use of Laboratory Animals, Revised (1986).

Tissue Disaggregation Protocol: Details for the mechanical disaggregation of rat liver tissue follows (yields aggregates of 1 to ≈15 cells) [10, 18]:

1) Working in a sterile 100 mm x 20 mm petri plate on ice, the lobes of the liver were minced into approximately 3 mm^3 sections using a pair of loose-jawed scissors. As the buffer became clouded with blood, it was decanted and replaced with fresh buffer.

2) A plunger from a 50 ml syringe was used to force sections of liver through a 1 mm nylon mesh (screen variety which may be purchased at a local hardware store), which had been stretched across a 250 ml beaker. Care was taken to keep the tissue wetted with HBSS, -Mg^{2+}, -Ca^{2+}, with EGTA at all times. Tissue which had adhered to the underside of the mesh was carefully scraped off and added to the cell suspension.

3) The first suspension of coarsely disaggregated tissue was then forced through a 273 μm nylon mesh which had been streched across a 250 ml beaker.

4) The cell suspension was transferred to sterile, disposable 50 ml centrifuge tubes, and centrifuged for 4 min at 100Xg, 4° C to pellet whole cells.

5) Pellets were then suspended in 10 - 15 ml of NH_4Cl buffer (see appendix A) and incubated on ice for 8 min. The tubes were slowly filled with HBSS, -Mg^{2+}, -Ca^{2+}, with EGTA [9].

6) Cells were centrifuged again at 100Xg, 4° C for 4 min. The supernatant was discarded leaving pellets of relatively pure liver cells.

7) Pellets were then washed a final time in L-15A medium (see appendix A).

ELISA protocol: The following are techniques recommended by J. S. Garvey (personal communication); the dilution (0.1 M carbonate-bicarbonate buffer, pH 9.6), wash and substrate buffer formulas are listed in appendix A.

1) In all of the assays, 200 µl of either standard antigen dilutions (RSA or KLH) or spent media dilutions were added to each of the test wells in a 96 well micro-titer plate. The outer wells were filled with 200 µl of wash buffer and used as non-specific binding controls. The plates were covered with adhesive film covers and incubated for 12 - 15 hrs at 4° C.

2) Plates were thoroughly washed by: a) "snap" movements of the inverted plates 2 to 3 times to empty the wells, b) rapping the plates against a cloth-covered counter top until no more wet spots were left on the cloth, c) filling the plates with 200 µl of wash buffer, and 4) allowing the covered plates to set at room temp for 5 min. This procedure was repeated twice more, extending the time that the wash buffer remained in the wells from 5 to 15 min on the third wash.

3) 100 µl of primary antibody diluted in wash buffer (anti-RSA diluted 1:800, or anti-KLH diluted 1:200), was then added to all the wells, the plates were covered with adhesive film, and incubated for 1 hr at 37° C. At the end of this period, the plates were washed 3 X as described above.

4) 100 µl of alkaline phosphatase-conjugated anti-IgG (diluted 1:1000) was added to each of the wells. The plates were covered with adhesive film, incubated for 1 hr at 37° C, and washed 3 X.

5) 150 µl of substrate buffer was added to each of the wells, and the plates were covered and incubated at 37° C for 30 min. The addition of substrate buffer to each plate was spaced 5 min apart to allow time to read one plate, and then set up the plate-reader for the subsequent plate.

6) The concentration of albumin in the wells was quantitated according to the absorbance of the hydrolyzed substrate at 405 nm.

Fixation, dehydration and embedding protocol: The following protocol was used for the preparation of tissue culture samples for light microscopy and vesicle suspensions for electron microscopy [12].

1) Samples were cut into ≈3 - 7 mm^3 pieces, fixed in.2.5% glutaraldehyde/2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, with 1 mM $CaCl_2$ for 30 min at room temperature, further cut into 1 - 2 mm^3 pieces and treated in the same fixative for 5 hours at 4° C (vesicles in agar were fixed for 48 hrs at 4° C).

2) The samples were washed 3X for 5 min each in the cacodylate buffer, and then post fixed in 1% osmium tetroxide in cacodylate buffer for 4 hour on ice. The samples were washed 3X as described above and then covered with distilled water.

3) Fixed samples were then dehydrated in an ethanol series (15%, 30%, 50%, 70%, 90% and 100% 3 X). Samples were kept in each of the ethanol solutions for a minimum of at least 15 min.

4) The ethanol was replaced with propylene oxide (the resin solvent) by treating the samples in the following ethanol: propylene oxide ratios, 3:1, 1:1, 1:3 (15 min each step) and then 100% propylene oxide (1 hr).

5) Epon 812 was infiltrated by incubating at room temp in a desiccator the samples in the following mixtures of propylene oxide: Epon 812 4:1 (2 hrs), 1:1 (4 hrs), 1:4 (4 hrs), 100% Epon 812 (overnight).

6) The samples were placed in molds, and incubated at 65° C for ≈70 hrs.

7) Sections were cut on a Sorvall Porter-Blum 2B ultramicrotome, using glass knives.

Agar embedment of vesicles: The collection and agar embedment of vesicles [23] are both described.

1) The disposable centrifuge tubes in which the vesicles were stored were shaken on a Vortex mixer to resuspend anything that may have settled out of solution, and then centrifuged at 100Xg for 5 min, 4° C to pellet any large cellular debris.

2) The supernatant was transferred to sterile, 30 ml Oak Ridge centrifuge tubes, and centrifuged at 22,000Xg, 4° C, for 40 min in a Beckman Spinco L ultracentrifuge using a type 30 angle-bucket rotor head.

3) The supernatant was carefully removed, the pellet washed in 1 ml of sterile filtered PBS, and resuspended in 1 ml of sterile filtered PBS.

4) The vesicle suspension was then warmed in a 45° C water bath and mixed with 0.15 ml of 7% noble agar which had been adjusted to the same temperature. The agar mixture was immediately poured on a chilled microscope slide, allowed to set, and cut into 1 mm^3 cubes with a sharp razor blade.

Immunolabelling of ultrathin sections: Immunolabelling of thin-sections was accomplished by floating the grids section-side down on drops of the appropriate reagents. The reagents were kept in para-film lined wells of a 24 well micro-titer plate in order to cut down on space and vessels used during the immunolabelling procedure. All steps were carried out at room temperature, and in accordance with the instructions provided by the gold-conjugated antibody manufacturer.

1) After the sections had been rinsed in dH_2O for 10 min, they were transferred to a saturated sodium metaperiodate solution for 1 hr. This extensive etching period was necessary to displace enough osmium tetroxide to allow reaction between antibodies and antigens of interest.

2) The sections were then jet-washed with dH_2O (sections were held in forceps while water from a pasteur pipette was sprayed down the tines of the forceps and over the grid), rinsed in 0.1 M HCl for 10 min, and rinsed again in dH_2O for 5 min.

3) The grids were incubated with 5% normal rabbit serum for 15 min to help determine the non-specific binding expected for the rabbit-anti-KLH antiserum.

4) Grids were transferred to the primary antibody solution for 1 hr. The primary antibody solution contained 1 part anti-KLH antisera (diluted 1:1000 in PBG), and 1 part of either monoclonal anti-MHC-I or monoclonal anti-MHC-II (also diluted 1:1000 in PBG). Because the 5 nm gold marker was conjugated to anti-rabbit IgG (and so was the marker for KLH), and the 10 nm gold was conjugated to anti-mouse IgG (making it the marker for MHCs), the sections could be assayed simultaneously for KLH and one of the MHCs.

5) Sections were washed 2X in PBG for 5 min each time, and incubated for 2 hr in a mixture of the two gold conjugated antibodies (diluted 1:25 in PBG, and supplemented with 1% normal goat serum).

6) The sections were then washed 2X in PBG for 5 min, 2X in PBS for 5 min and post-fixed with 2% glutaraldehyde (electron microscopy grade in PBS) for 15 min.

7) After 2 final 5 min washes in dH_2O, the grids were counterstained with uranyl acetate and lead citrate. The staining procedure and reagents were the same as those described above for non-immunolabelled sections, except that staining times were increased to 25 min. The extended staining time was used to try and overcome the membrane bleaching effect caused by periodic acid etching.

Quantigold assay: The Quantigold assay is a colorimetric assay which is much more sensitive and much less time dependent than either the biuret or Lowrey assays. The following protocol was based on instructions provided by the manufacturer.

1) 10 µl of protein diluted in PBS (series diluted from 1:100, to 1:1000) was mixed with 0.8 ml of Quantigold reagent in disposable, semi-micro cuvettes. Cuvettes were used only one time, to prevent cross-contamination.

2) After a 30 min incubation period (room temperature), the samples were checked for absorbance at 595 nm. Because of the color stabilization that occurs with this reagent after 30 min, there was no need to space the samples so that each was incubated for exactly the same period of time.

3) A KLH standard was used to construct a standard curve.

LITERATURE CITED

1. **Bellanti, J. A.** 1975. Adverse affects of viral vaccines, p. 327-339. *In* A. L. Notkins (ed.), Viral Immunology and Immunopathology. Academic Press, New York, NY.

2. **Benacerraf, B., L. D. Falo, Jr., and K. L. Rock.** 1988. Processing of native antigen by accessory cells and presentation of membrane-bound MHC-associated antigen to specific T cells, p. 1-11. *In* B. Pernis, S. C. Silverstein, and H. J. Vogel (ed.), Processing and presentation of antigens. Academic Press, Inc., New York, NY.

3. **Burdick, B. A., and J. R. Schaeffer** 1988 Production of specialty chemicals using immobilized whole cells in spiral-wound bioreactors: I. aspartic acid and alanine production using cells immobilized in gelatin on nylon mesh. Biotechnol. Bioeng. **31**: 390-395.

4. **Cai, Z., Z. Shi, M. Sherman and A. M. Sun.** 1989. Development and evaluation of a system of microencapsulation of primary rat hepatocytes. Hepatology. **10:** 855-860.

5. **Chambard, M., J. Gabrion and J. Mauchamp.** 1981. Influence of collagen gel on the orientation of epithelial cell polarity: follicle formation from isolated thyroid cells and from preformed monolayers. J. Cell Biol. **91:** 157-166.

6. **Chanock, R. M., D. D. Richman, B. R. Murphy, S. B. Spring, T. J. Schnitzer and L. S. Richardson.** 1975. Current approaches to immunoprophylaxis, p. 291-316. *In* A. L. Notkins (ed.), Viral Immunology and Immunopathology. Academic Press, New York, NY.

7. **Chung, K., P. Walter, G. W. Aponte and H. H. Moore.** 1989. Molecular sorting in the secretory pathway. Science. **243**: 192-197.

8. **Clark, J. M. Jr., and R. L. Switzer.** 1977. Experimental Biochemistry, 2nd Edition, p. 99. W.H. Freeman and Company, New York.

9. **Coleman, R. M., M. F. Lombard, R. E. Sicard, and N. J. Rencricca.** 1989. Fundamental Immunology, p. 83 - 103, 135, 340 - 344, 399. Wm. C. Brown Publishers, Dubuque, Iowa.

10. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic Methods in Molecular Biology, p. 45, 100, 158, 348-352. Elsevier, New York.

11. **Davis, R. A., S. C. Engelhorn, S. H. Pangburn, D. B. Weinstein and D. A. Steinberg.** 1979. Very low density lipoprotein synthesis and secretion by cultured rat hepatocytes. J. Biol. Chem. **254:** 2010-2016.

12. **Dawes, C. J.** 1988 Introduction to Biological Electron Microscopy: Theory and Techniques, p. 113-114, 188, 208. Ladd Research Industries, Inc., Publisher, Burlington, VT.

13. **Dunn, J. C. Y., M. L. Yarmush, H. G. Koebe, and R. G. Tompkins.** 1989. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configurgation. FASEB Jour. **3:** 174-177.

14. **Elsdale, T., and J. Bard**. 1972. Collagen substrata for studies on cell behavior. J. Cell Biol. **54**: 626-637.

15. **Emerson, S. G., and R. E.Cone**. 1981. I-K^K and H-$2K^K$ antigens are shed as supramolecular particles in association with membrane lipids. J. Immunol. **127**: 482-486.

16. **Farrant, J., C. A. Walter, H. Lee, G. J. Morris and K. J. Clarke.** 1977. Structural and functional aspects of biological freezing techniques. J. of Microsc. (Oxf.). **111**: 17-34.

17. **Fraenkel-Conrat, H., P. C. Kimball and J. A. Levy.** 1988. Virology, 2nd edition, p. 187, 252, 272. Prentice Hall, Englewood Cliffs, NJ.

18. **Freshney, I. R.** 1987. Culture of Animal Cells; A Manual of Basic Technique, 2nd edition, p. 66-75, 85 - 88, 124 - 126. Alan R. Liss Inc, New York, NY.

19. **Garvey, J. S.** 1961. Separation and *in vitro* culture of cells from liver tissue. Nature. **191:** 972-974.

20. **Garvey, J. S. and D. H. Campbell.** 1966. Autoradiographic investigation of tissues after primary and multiple antigenic stimulation of rabbits. Nature. **209:** 1201-1202.

21. **Garvey, J. S., N. E. Cremer, and D. H. Sussdorf.** 1977. Methods in Immunology; A Laboratory Text for Instruction and Research; 3rd edition, p. 54-55, 135-139, 445, 524. Benjamin/Cummings Publishing Co., Reading Mass.

22. **Garvey, J. S. and E. B. Reilly.** 1979. Studies of antigen-RNA and immunity. Mol. Cell. Biochem. **24:** 45-62.

23. **Glauert, A. M.** 1965. The Fixation and Embedding of Biological Specimens, p. 165-212. *In* D. H. Kay (ed.), Techniques for Electron Microscopy, 2nd Edition. F. A. Davis Company, Philadelphia, PA.

24. **Gottlieb, A. A.** 1969. Studies on the binding of soluble antigens to a unique ribonucleoprotien fraction of macrophage cells. Biochemistry. **8:** 2111-2116.

25. **Gottlieb, A. A.** 1969. Macrophage ribonucleoprotien: nature of the antigenic fragment. Science. **165**: 592-594.

26. **Guguen-Guillouzo, C., B. Clement, G. Baffet, C. Beaumont, E. Morel-Chany, D. Glaise and A. Guillouzo.** 1983. Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. Exp. Cell Res. **143**: 47-54.

27. **Guigoz, Y. P. Werffeli, D. Favre, M. Juillerat, R. Wellinger and P. Honneger**. 1987. Aggregate cultures of foetal rat liver cells: development and maintenance of liver gene expression. Biol. Cell. **60:** 163-172.

28. **Heil, M. F., A. D. Dingman and J. S. Garvey.** 1984. Antigen handling in ageing. III. Age-related changes in antigen handling by liver parenchymal cells. Mech. Ageing Dev. **26:** 327-40.

29. **Herlyn, M., U. Rodeck and H. Koprowski.** 1987. Shedding of human tumor associated antigens *in vitro* and *in vivo*. Adv. Cancer Res. **49:** 189-221.

30. **Horne, R. W.** 1965. Negative staining methods, p. 328-355. *In* D. H. Kay (ed.), Techniques for Electron Microscopy, 2nd edition. F. A. Davis Company, Philadelphia, PA.

31. **Horstman, D. M.** 1978. Immunization against viral infections, p. 286. *In* H. Rothschild, F. Allison, Jr., and C. Howe (ed.), Human Diseases Caused by Viruses. Oxford University Press, New York, NY.

32. **Institute of Health. Division of Health Promotion and Disease Prevention, and Division on International Health.** 1986. New Vaccine Development; Establishing Priorities, vol 2, Diseases of Importance in Developing Countries, p. 5-15. National Academy Press, Washington, D. C.

33. **Ishag, M., and R. Ali.** 1983. Purification and identification of antigenic polypeptides of Sm and RNP antigens of goat liver. Biochem. Biophys. Res. Commun. **114:** 564-570.

34. **Isom, H., I. Georgieff, M. Salditt-Georgieff and J. E. Darnell, Jr.** 1987. Persistence of liver-specific messenger RNA in cultured hepatocytes: different regulatory events for different genes. J. Cell Biol. **105**: 2877-2885.

35. **Isom, H. C., T. Secott, I. Georgoff, C. Woodworth and J. Mummaw.** 1985. Maintenance and differentiated rat hepatocytes in primary culture. Proc. Nat. Acad. Sci. USA. **82**: 3252-3256.

36. **Jauregui, H. O., P. N. McMillan, J. Driscoll, S. Naik.** 1986. Attachment and long term survival of adult rat hepatocytes in primary monolayer cultures: comparison of different substrata and tissue culture media formulation. In Vitro. **22**: 13-17.

37. **Jones, O. T., J. P. Earnest and M. G. McNamee.** 1987. Solubilization and reconstitution of membrane proteins, p. 154-155. *In* J. B. C. Findlay and W. H. Evans (ed.), Biological Membranes -- A Practical Approach. IRL Press, Oxford, England.

38. **Kabir, S., D. C. Rosenstreich and S. E. Mergenhagen.** 1978. Bacterial endotoxins and cell membranes. *In* J. Jeljaszewicz and T. Wadstrom, Bacterial Toxins and Cell Membranes. Academic Press, New York, NY.

39. **Kamat, V. B., and D. F. H Wallach.** 1965. Seperation and partial purification of plasma-membrane fragments from erlich ascites carcinoma microsomes. Science. **148**: 1343-1345.

40. **Kan, M., and I. Yamane.** 1984. Effects of Ferrous Iron and Transferrin on Cell Proliferation of Human Diploid Fibroblasts in Serum-Free Culture. In Vitro. **20:** 89-94.

41. **Kleinman, H. K., R. J. Klebe and G. R. Martin.** 1981. Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. **88**: 473-485.

42. **Kreamer, B. L., Staecker, J. L., Sawanda, N., Sattler, G. L., Hsia, M. T. S., and H. Pitot.** 1986. Use of low speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro. **22**: 201-211.

43. **Lee, M. and J. R. Debro.** 1963. The application of gel filtration to the measurement of the binding of phenol red by human serum protein. J. Chromatogr. **10**: 68-71.

44. **Lehninger, A. L.** 1978. Biochemistry: The Molecular Basis of Cell Structure and Function. Worth Publishers, Inc., New York, NY.

45. **Maher, J. J.** 1988. Primary hepatocyte culture: is it home away from home? Hepatology. **8**: 1162-1166.

46. **Mesnil, M., J., Fraslin, C. Piccoll, H. Yamasaki, and C. Guguen-Guillouzo.** 1987. Cell contact but not junctional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions. Exp. Cell Res. **173**: 524-533.

47. **Michalopoulos, G., and H. C.Pitot.** 1975. Primary culture of parenchymal liver cells on collagen membranes. Exp. Cell Res. **94**: 70-75.

48. **Miyazaki, M, Y. Suzuki, M. Oda, A. Kawai, L. Bai, and J. Sato.** 1989. Improved maintenance of adult rat hepatocytes in a new serum-free medium in the presence or absence of barbiturates. In Vitro. **25**: 839-848.

49. **Niederwieser, A., and G. Pataki.** 1973. New Techniques in Amino Acid, Peptide and Protein Analysis, p. 227-269. Ann Arbor Science Publisher, Ann Arbor MI.

50. **Oliver, I. T., A. M. Edwards and H. C. Pitot.** 1978. Hormonal regulation of phospho*enol* pyruvate carboxykinase in primary cultures of adult-rat liver parenchymal cells. Eur. J. Biochem. **87**: 221-227.

51. **Oliver, J. M., J. Seagrave, R. F. Stump, J. R. Pfieffer, and G. G. Deanin.** 1988. Signal transduction and cellular response in RBL-2H3 mast cells, p. 185-245. *In* E. L. Becker (ed.), Membrane Activation in Immunologically Relevant Cells. Karger, New York, NY.

52. **Perry, S. D. and D. I. C. Wang.** 1989. Fiber bed reactor design for animal cell culture. Biotech. Bioeng. **34**: 1-9.

53. **Pettipher, G. L.** 1983. The Direct Epifluorescent Technique (for the rapid enumeration of micro-organisms) p. 86-99. Research Studies Press, LTD, Letchworth, Hertfordshire, England.

54. **Roth, J.** 1984. The protein A-gold technique for antigen localisation, p. 113-121. *In* J. M. Polak and I. M. Varndell (ed.), Immunolabelling for Electron Microscopy, Elsevier Science Publishing Company, Inc., New York, NY.

55. **Scharschmidt, B. F., J. R. Lake, E. L. Renner, V. Licko and R. W. Van Dyke.** 1986. Fluid phase endocytosis by cultured rat hepatocytes and perfused rat liver: implications for plasma membrane turnover and vesicular trafficking of fluid phase markers. Proc. Natl. Acad. Sci. USA. **83:** 9488-9492.

56. **Schor, S.** 1968. Fundamentals of Biostatistics. G. P. Putnam's Sons, New York.

57. **Shapiro, L. E. and N. Wagner.** 1989. Transferrin is an autocrine growth factor secreted by Reuber H-35 cells in serum-free culture. In Vitro. **25:** 650-654.

58. **Sirica, A. E., W. Richards, Y. Tsukada, C. A. Sattler and H. C. Pitot.** 1979. Fetal phenotypic expression by adult rat hepatocytes on collagen gel/nylon meshes. Proc. Natl. Acad. Sci. USA. **76**: 283-287.

59. **Skogh, T., R. Blumhoff, W. Eskild and T. Berg.** 1985. Hepatic uptake of circulating IgG immune complexes. Immunology. **55:** 585-594.

60. **Sundaram, P. V., and W. E.,Hornby**. 1970. Preparation and properties of urease chemically attached to nylon tube. FEBS Lett. **10**: 325-327.

61. **Szakal, A. K., K. L. Holmes and J. G. Tew.** 1983. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells, including cells with dendritic morphology. J. of Immunol. **131:** 1714-1727.

62. **Szakal, A. K., M. H. Kosco and J. G. Tew.** 1988. A novel in vivo follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen-processing cells. J. of Immunol. **140**: 341-353.

63. **Szakal, A. K., M. H. Kosco and J. G. Tew.** 1989. Microanatomy of lymphoid tissue during humoral immune responses: structure function relationships. Annu. Rev. Immunol. **7:** 91-109.

64. **Taylor, D. D. and P. H. Black.** 1985. Inhibition of macrophage Ia antigen expression by shed plasma membrane vesicles from metastatic murine melanoma lines. J. Nat. Cancer Inst. **74**: 859-867.

65. **Taylor, D. D., and P. H. Black**. 1986. Shedding of plasma membrane fragments -- neoplastic and developmental importance, p. 33-59. *In* M. S. Steinberg (ed.), Developmental Biology; A Comprehensive Synthesis, Volume 3. The Cell Surface in Development and Cancer. Plenum Press, New York.

66. **Trippet, K. P.** 1988. The immunological role of the liver after *in vivo* stimulation by antigen: antigen presentation by purified liver cell populations. Masters Thesis. Syracuse University, Syracuse, NY.

67. **Umbreit, W. W., R. H. Burris and J. F. Stauffer.** 1951. Manometric Techniques and Tissue Metabolism, pp 110-111. Burgess Publishing Co., Minneapolis, MN.

68. **Van Blitterswijk, W. J., G. De Veer, J. H. Krol and P. Emmelot.** 1982. Comparative lipid analysis of purified plasma membranes and shed extracellular membrane vesicles from normal murine thymocytes and leukemic GRSL cells. Biochim. Biophys. Acta. **688**: 495-504.

69. **Yamada, K. M.** 1983. Cell surface interactions with extracellular materials. Ann. Rev. Biochem. **52:** 761-799.

70. **Yamazaki, K. and N. F. LaRusso.** 1989. The liver and intracellular digestion: how lever cells eat! Hepatology. **10:** 877-886.

BIOGRAPHICAL DATA

Name: Timothy Francis Moshier

Date and Place of Birth: August 21, 1959
Fulton, New York

Elementary School: Fair Haven Elementary School
Fair Haven, New York
Graduated 1971

High School: Red Creek Central High School
Red Creek, New York
Graduated 1977

College: State University of New York, College at Oswego
Oswego, New York
B. A. 1981

Graduate Work: Syracuse University
Syracuse, New York.